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The role of Ca^{2+} signalling and bile acids in the biology of Barrett's oesophagus and oesophageal adenocarcinoma

Thesis submitted to the National University of Ireland, Cork, in fulfilment of the requirements for the Master's Degree by Research

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TABLE OF CONTENTS

CHAPTER 3.

Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, at either University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property.

Detailed author contributions can be found included in each research chapter.

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Ms. Alana Cutliffe

17th July 2025

Date

I. List of figures

INTRODUCTION

CHAPTER 2.

CHAPTER 3.

DISCUSSION

II. List of tables

INTRODUCTION

CHAPTER 3

III. List of abbreviations

LIST OF ABBREVIATIONS IN THE CURRENT WORK	
ABBREVIATION	**FULL NAME**
$[Ca^{2+}]_c$	Cytoplasmic Ca^{2+} concentration
5-FU	Fluorouracil
8OHdG	8-Hydroxydeoxyguanosine
ACCN4	Acid-sensing ion channel 4 gene
AJCC	American Joint Committee on Cancer
ALDH1A2	Aldehyde dehydrogenase 1 family member A2 gene
ANOVA	Analysis of variance
AP-1	Activator protein 1
ASIC	Acid-sensing ion channel
ATOH1	Atonal bHLH transcription factor 1
ATP2C2	Secretory pathway Ca^{2+} ATPase gene
ATP6V0A1	ATPase H+ transporting V0 subunit A1 gene
ATP6V0A2	ATPase H+ transporting V0 subunit A2 gene
ATP6V0A4	ATPase H+ transporting V0 subunit A3 gene
ATP6V0B	ATPase H+ transporting V0 subunit B gene
ATP6V0C	ATPase H+ transporting V0 subunit C gene
BA	Bile acid
Bcl-2	B-cell leukaemia / lymphoma 2 protein
BO	Barrett's oesophagus
c-Fos	Cellular oncogene c-Fos
CA	Cholic acid
CA1	Carbonic anhydrase 1
CA10	Carbonic anhydrase 10
CA11	Carbonic anhydrase 11
CA12	Carbonic anhydrase 12
CA13	Carbonic anhydrase 13
CA14	Carbonic anhydrase 14
CA2	Carbonic anhydrase 2

Ca^{2+}	Calcium
CA3	Carbonic anhydrase 3
CA4	Carbonic anhydrase 4
CA6	Carbonic anhydrase 6
CA7	Carbonic anhydrase 7
CA8	Carbonic anhydrase 8
CA9	Carbonic anhydrase 9
CACNA1D	Calcium voltage-gated channel subunit alpha 1D
CACNA1G	Calcium voltage-gated channel subunit alpha 1G
CACNA2D4	Calcium voltage-gated channel auxiliary subunit alpha 2 delta 4
CALR	Calreticulin
CAMK	Calcium / calmodulin dependent protein kinase
CC3	Cleaved caspase 3
CD4+ T cells	Cluster of differentiation 4 positive T lymphocytes
CD8+ T cells	Cytotoxic T lymphocytes
CDCA	Chenodeoxycholic acid
CDKN2A	Cyclin dependent kinase inhibitor 2A
CDX1	Caudal type homeobox 1
CDX2	Caudal type homeobox 2
CF	Cisplatin and fluorouracil
CFTR	Cystic fibrosis transmembrane conductance regulator gene
COX-2	Cyclo-oxygenase 2
CREB	CAMP responsive element binding protein
CROSS	Chemoradiotherapy for Oesophageal Cancer followed by Surgery Study
CX	Cisplatin and capecitabine
CXCL-8	CXC motif chemokine ligand 8
CYP3A	Cytochrome P450 family 3 subfamily A
CYP7A1	Cytochrome P450 family 7 subfamily A member 1
DCA	Deoxycholic acid
DHP	Dihydropyridine
DLL-1	Delta-like canonical notch ligand 1

DNA	Deoxyribonucleic acid
EA	Extracellular acid
ECF	Extracellular fluid
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
FLOT	Fluorouracil, leucovorin, oxaliplatin and docetaxel
FOLFOX	Leucovorin, fluorouracil and oxaliplatin
FXR	Farnesoid X receptor
GCA	Glycocholic acid
GCDCA	Glycochenodeoxycholic acid
GDCA	Glycodeoxycholic acid
GI	Gastro-intestinal
GORD	Gastro-oesophageal reflux disease
GPR	G-protein coupled receptor
GPR132	G-protein coupled receptor 132
GPR4	G-protein coupled receptor 4
GPR65	G-protein coupled receptor 65
GPR68	G-protein coupled receptor 68
GPX1	Glutathione peroxidase 1
GRIN2D	N-methyl-D-aspartate receptor subunit 2D
HCl	Hydrochloric acid
HER2	Human epidermal growth factor receptor 2
Hes1	Hairy and enhancer of split 1
HGD	High-grade dysplasia
HVCN1	Hydrogen voltage-gated channel 1
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
IP_3	Inositol 1,4,5-trisphosphate
IP_3R	Inositol 1,4,5-trisphosphate receptor

IκBα	NF-κB inhibitor alpha
JPH	Junctophilin
KLF4	Krüppel-like Factor 4
Kv10.1	Voltage-gated potassium channel subfamily H member 1
LCA	Lithocholic acid
LGD	Low-grade dysplasia
LOH	Loss of heterozygosity
MAIT cells	Mucosal-associated invariant T cells
MCOLN1	Mucolipin TRP cation channel 1
MCOLN2	Mucolipin TRP cation channel 2
MCOLN3	Mucolipin TRP cation channel 3
Minority MOMP	Mitochondrial Outer Membrane Permeabilization
miR-145	microRNA-145
MOT	Multiplicity of testing
MUC2	Mucin 2 gene
MUC5AC	Mucin 5AC gene
NAT	Normal adjacent tissue
NCX	Na^+ / Ca^{2+} exchanger
NDBO	Non-dysplastic Barrett's oesophagus
NF-κB	Nuclear factor kappa B
NFAT	Nuclear factor of activated T-cells
NICE	National Institute for Health and Care Excellence
Notch 1	Neurogenic locus notch homolog protein 1
NOX5-S	NADPH Oxidase 5
OAC	Oesophageal adenocarcinoma
OC	Oesophageal cancer
OCCAMS	Oesophageal cancer clinical and molecular stratification
OCT4	Octamer-binding transcription factor 4
ORAI1	Orai calcium release activated calcium modulator 1 gene
OrSCC	Oral squamous cell carcinoma
OSCC	Oesophageal squamous cell carcinoma

p-H2AX	phospho-H2A histone family member X
p53	Transformation-related protein 53
PD-L1	Programmed cell death 1 ligand 1
PGE2	Prostaglandin E2
phospho-p38-MAPK	p38 mitogen-activated protein kinase
PLC	Phospholipase C
PM	Plasma membrane
PXR	Pregnane X receptor
QUIN	Quality Assessment Tool For In Vitro Studies
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
RYR	Ryanodine receptor
RYR1	Ryanodine receptor 1 gene
RYR2	Ryanodine receptor 2 gene
RYR3	Ryanodine receptor 3 gene
S1PR2	Sphingosine 1 phosphate receptor 2
SERCA1	Sarcoplasmic / endoplasmic reticulum Ca^{2+} ATPase 1
SERCA2B	Sarcoplasmic / endoplasmic reticulum Ca^{2+} ATPase 2B
SLC26A1	Solute carrier family 26 member 1
SLC26A10	Solute carrier family 26 member 10
SLC26A2	Solute carrier family 26 member 2
SLC26A3	Solute carrier family 26 member 3
SLC26A4	Solute carrier family 26 member 4
SLC26A5	Solute carrier family 26 member 5
SLC26A6	Solute carrier family 26 member 6
SLC26A7	Solute carrier family 26 member 7
SLC26A8	Solute carrier family 26 member 8
SLC26A9	Solute carrier family 26 member 9
SLC4A1	Solute carrier family 4 member 1
SLC4A10	Solute carrier family 4 member 10

SLC4A11	Solute carrier family 4 member 11
SLC4A2	Solute carrier family 4 member 2
SLC4A3	Solute carrier family 4 member 3
SLC4A4	Solute carrier family 4 member 4
SLC4A5	Solute carrier family 4 member 5
SLC4A7	Solute carrier family 4 member 7
SLC4A8	Solute carrier family 4 member 8
SLC4A9	Solute carrier family 4 member 9
SLC9A1	Solute carrier family 9 member A1
SLC9A2	Solute carrier family 9 member A2
SLC9A3	Solute carrier family 9 member A3
SLC9A4	Solute carrier family 9 member A4
SLC9A5	Solute carrier family 9 member A5
SLC9A6	Solute carrier family 9 member A6
SLC9A7	Solute carrier family 9 member A7
SLC9A8	Solute carrier family 9 member A8
SLC9A9	Solute carrier family 9 member A9
SMAD4	SMAD family member 4 gene
SOCE	Store-operated calcium entry
SPCA1	Secretory pathway Ca^{2+} ATPase 1
SPCA2	Secretory pathway Ca^{2+} ATPase 2
SR	Sarcoplasmic reticulum
STIM	Stromal interaction molecule
STROBE	Strengthening the Reporting of Observational Studies in Epidemiology
TCA	Taurocholic acid
TCDCA	Taurochenodeoxycholic acid
TDCA	Taurodeoxycholic acid
TGCA	The Cancer Genome Atlas
TGF-β	Transforming growth factor beta
TGR5	Takeda G-protein coupled receptor 5
Th2 cells	T helper 2 cells

TLCA	Taurolithocholic acid
TME	Tumour microenvironment
TNM	Tumour, nodes and metastases
TP53	p53 gene
TPC	Two-pore channel
TPM	Transcripts per million
TPPO	Triphenylphosphine oxide
TRP	Transient receptor potential channel
TRPA1	Transient receptor potential channel subfamily A member 1
TRPC4	Transient receptor potential channel subfamily C member 4
TRPC5	Transient receptor potential channel subfamily C member 5
TRPC6	Transient receptor potential channel subfamily C member 6
TRPM1	Transient receptor potential channel subfamily M member 1
TRPM2	Transient receptor potential channel subfamily M member 2
TRPM4	Transient receptor potential channel subfamily M member 4
TRPM5	Transient receptor potential channel subfamily M member 5
TRPM6	Transient receptor potential channel subfamily M member 6
TRPM8	Transient receptor potential channel subfamily M member 8
TRPV1	Transient receptor potential channel subfamily V member 1
TRPV2	Transient receptor potential channel subfamily V member 2
TRPV3	Transient receptor potential channel subfamily V member 3
TRPV4	Transient receptor potential channel subfamily V member 4
TRPV5	Transient receptor potential channel subfamily V member 5
TUDCA	Tauroursodeoxycholic acid
UDCA	Ursodeoxycholic acid
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
VGCC	Voltage-gated calcium channel
WHO	World Health Organization

IV. Abstract

Oesophageal adenocarcinoma (OAC) is highly prevalent in Ireland (1.8 per 100,000 persons), is on the rise and is associated with poor patient survival (5-year survival of less than 15%). There is a high rate of recurrence (up to 50% after an oesophagectomy) and the neoadjuvant chemotherapy response rate is 32%. A precursor condition, Barrett's oesophagus (BO), is associated with hydrochloric acid and bile acid (BA) reflux from the stomach. Little is known about how oesophageal cells interact with acidic and BA environments and remodel to form BO or OAC. Such effects may be mediated via Ca^{2+} signalling, but little work on the topic has been carried out to date. The aim of this study was to investigate the role of Ca^{2+} signalling (Research Objective A) and BA exposure (Research Objective B) in the development or prevention of BO and OAC. A transcriptomic analysis of 275 Ca^{2+} signalling genes was carried out on two independent OAC datasets (Research Objective A) and a systematic review (20 studies) was carried out on neutral-pH, BA exposure in the prevention and development of BO and OAC (Research Objective B). Of the 275 Ca^{2+}-toolkit genes analysed, 75 displayed consistent changes in expression between OAC and normal tissue in both datasets. The channel-encoding genes, *N*-methyl-*D*-aspartate receptor 2D (*GRIN2D*), transient receptor potential (TRP) ion channel classical or canonical 4 (*TRPC4*), and TRP ion channel melastatin 2 (*TRPM2*) demonstrated the greatest increase in expression in OAC in both datasets. Six highly expressed genes were associated with improved patient survival: voltage-gated Ca^{2+} channel subunit α 1D (*CACNA1D*), voltage-gated Ca^{2+} channel auxiliary subunit α2 δ4 (*CACNA2D4*), junctophilin 1 (*JPH1*), acid-sensing ion channel 4 (*ACCN4*), *TRPM5*, and secretory pathway Ca^{2+} ATPase 2 (*ATP2C2*). The six genes in question were also upregulated in advanced OAC tumor grades and nodal, metastatic stages. Key observations in the BA systematic review were that deoxycholic acid exerted effects on induction of BO and OAC through several potentially co-operating mechanisms, including oxidative stress, DNA damage, inflammation, proliferation, apoptosis, apoptotic resistance and angiogenesis. In BO, taurodeoxycholic acid was associated with oxidative-stress and DNA damage. Treatment of non-malignant human oesophageal cells and BO cells with ursodeoxycholic acid in vitro resulted in a reduction of deoxycholic-acid-induced inflammation. In conclusion, both Ca^{2+}

signalling and BA exposure may play a role in the development of BO and OAC. Ca^{2+} signalling is relatively understudied in cancer and may provide novel therapeutic avenues and a reduction in side-effects. BAs could be targeted therapeutically, through medication, bacterial supplementation, or lifestyle modifications. The possibility of a reduced side-effect therapy and lifestyle interventions are of great appeal to cancer patients. The results presented in the current study lay the groundwork for highly informed laboratory research hypotheses.

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VI. Publications

Peer-reviewed publications

Cutliffe AL, McKenna SL, Chandrashekar DS, Ng A, Devonshire G, Fitzgerald RC, et al. Alterations in the Ca^{2+} toolkit in oesophageal adenocarcinoma. Explor Target Antitumor Ther. 2021;2:543-75. https://doi.org/10.37349/etat.2021.00063

The above study forms the basis of Chapter 2 in the current work.

CHAPTER ONE: INTRODUCTION

1. Oesophageal Cancers (OCs)

1.1. Types and epidemiology

Oesophageal cancers (OCs) arise from the uncontrolled growth of cells, forming a tumour, in various parts of the oesophagus of the human body [1]. Histologically, there are two distinct forms of OC: squamous cell carcinoma (OSCC), derived from squamous epithelial cells normally in the upper part of the oesophagus, and adenocarcinoma (OAC), arising from glandular cells in the lower part of the oesophagus [1], [2]. OSCC is most prevalent in South Africa, East Africa, East Asia and Central Asia, whereas OAC is the most common type in North America, Western Europe and Australia [3], [4], [5]. In brief, OSCC arises from the dysplasia (the growth of abnormal cells) of oesophageal squamous epithelia [5]. By contrast, OAC arises from the metaplasia (substitution of one differentiated somatic cell type to another in the same tissue) of squamous epithelia to columnar and goblet (mucous-secreting) cells, followed by dysplasia and hyperplasia (increased rate of growth) [5]. A precursor condition along this spectrum to OAC development is a condition called Barrett's oesophagus (BO) [6]. The major risk factors for OSCC include smoking and alcohol consumption, whereas the major risk factors for OAC include BO, gastro-oesophageal reflux disease (GORD), achalasia (the failure of the lower oesophageal sphincter to open), male gender and central obesity [5]. Symptoms of OCs include: difficulty swallowing; persistent heartburn; pain in the breastbone, back or throat; weight loss; poor appetite; ongoing cough; and vomiting or regurgitation of food [5].

OCs, grouped as OSCC, OAC and "histology not otherwise specified", are the sixth-leading cause of cancer-related deaths worldwide [7]. OCs are the eighth most common cancer globally [7] and the thirteenth most common cancer in Ireland [8]; Ireland also has the third highest incidence in the European Union [9]. In Europe, 5 cases per 100,000 individuals are diagnosed each year [7]; in Ireland, the mean number of new cases diagnosed each year is 182 in women and 301 in men [8]. OAC is the most common form of oesophageal cancer in Ireland, where it accounts for 50.9% of all OCs diagnosed from 2010 to 2014 [10]. The age-standardized 5-year net

survival for OCs (2010–2014) was 21.9% for Ireland [2], 16.2% for the UK [2], and 20% for the United States [3].

OAC is the most common form in Northern (incidence of 3.3 per 100,000 persons) and Western Europe (1.8 per 100,000 persons) [11]. Eighty-five thousand cases of OAC were diagnosed were diagnosed in 2018 [4]. Globally, the incidence of OAC has increased more than 6-fold in the last thirty years [12]. It is also predicted that new OAC cases will rise by 82% for the nineteen-year period of 2021 to 2040 [13]. OAC has an overall, five-year survival of less than 15% in most populations [14], [15], with one study reporting values as low as 8% in the UK [15]. Causes of poor prognosis include late diagnosis (lack of symptoms at earlier stages of the disease), incomplete resection of tumours, and resistance to chemotherapeutic and radio-therapeutic interventions [16], [17], [18], [19]. Even with a full curative oesophagectomy, the rate of recurrence of OCs is up to 50% [20]. The neo-adjuvant chemotherapy response rate for OAC is 32% [21]. The relatively high rate of resistance to current chemotherapies raises the need to develop novel chemotherapeutics, looking beyond conventional mechanisms [21]. This thesis will focus on OAC, rather than OSCC .

1.2. Risk factors for oesophageal adenocarcinoma (OAC)

Risk-factors for OAC include age, gender, racial, bacterial-infection, pharmacological and lifestyle-related factors (Table 1.1). The top three risk factors (BO, *H. pylori* infection and GORD) are associated (positively or negatively) with acid reflux, bile reflux or both [6], [22], [23]. Little is known, however, about how oesophageal cells detect and respond to these stimuli [24].

Table 1.1. Risk factors for oesophageal adenocarcinoma (OAC)

OAC RISK FACTOR	**DETAILS**
Age	Average age at diagnosis: 72 years [25]
Gender	OAC is 9 times more common in men than in women [26]

Race	OAC is higher in whites, compared to black and Asian populations [27]
Bacterial infection	Those with *Helicobacter pylori* infection have a 41-44% reduced risk of OAC [28], [29]
Smoking	Increased risk of OAC development [30] Smoking cessation associated with a decreased risk of OAC development [30]
Achalasia (failure of the lower oesophageal sphincter to relax during swallowing)	Increased risk of OAC development [31], [32]
Gastro-oesophageal reflux disease (GORD)	Increased risk of OAC development [33]
Barrett's Oesophagus (BO)	40-50 fold increased risk of OAC development, compared to the general population [6]
High Body Mass Index	Increased risk, partly due to the increased likelihood of having GORD [34], [35]
Physical Activity	The "physically active" (definition varied per study in the meta-analysis) had a 32% reduced risk of developing OAC [36]
Non-steroidal anti-inflammatory drug use	Reduced risk of OAC development [37]
Diet quality	Increased risk of OAC development with low Vitamin-C intake, low Vitamin-E intake, low total-carotenoid intake, low selenium intake, low folate intake, high total-fat intake and high saturated-fat intake [38], [39], [40]

1.3. Family history and OAC risk

OAC most frequently occurs in European and North American populations. A family-history study (a cohort study from Sweden) has been carried out and has noted that a family history of OC (grouped as OSCC and OAC) or OSCC increased OAC risk [41]. All OAC-related, family-history studies (from Europe, North America or elsewhere) acknowledge that family-history associations could be due to shared environmental influences of families, as opposed to genetic factors [41], [42].

1.4. Key driver gene mutations in OAC

Ek et al. noted that a predisposing genetic background (many germline genetic variants of small effect) accounted for 25% of OAC cases [43]. Other authors state that the vast majority (greater than 90%) of OAC cases are sporadic and caused by somatic (post-conceptional) mutations [44]. OAC is the top-third, most frequently mutated cancer [45]: the cancer has, on average, 10 mutations per megabase of DNA and the number of mutations increase from BO to low-grade dysplasia (LGD), from LGD to high-grade dysplasia (HGD), from HGD to OAC [46]. Loss of heterozygosity (LOH) is a specific type of somatic mutation during which there is a loss of one parental copy of a gene or group of genes [46]. Table 1.2 details the association between LOH mutations and OAC development.

Table 1.2. Association between loss of heterozygosity (LOH) mutations and oesophageal adenocarcinoma (OAC) development

CHROMOSOMAL ARM	**LOH MUTATION FREQUENCY**	**SAMPLE OAC "DRIVER GENE"**	**GENE DETAILS**
17p	96% [47]	*TP53* [47]	Encodes p53, a tumour-suppressor protein [46] LOH mutations contribute to the progression of non-

			dysplastic BO to BO with low-grade dysplasia [46]
18q	70% [47]	*SMAD4* [47]	Encodes Smad4, a tumour-suppressor protein [46] LOH mutations contribute to the worsening of OAC [46]
11p	61% [47]	*MUC5AC* [47]	Encodes Mucin 5AC, a mucin-producing protein [48]. Expression downregulated in OAC samples [49]

Having a combination of risk-increasing mutations is more harmful than having a single mutation, given that no single mutation has demonstrated to increase the risk of OAC by more than 20% [43], [50]. Indeed, Kunzmann et al. illustrated that the inclusion of genetic information (18 recognised genetic variants, including variants of the Cystic Fibrosis (*CFTR*) gene and an alcohol dehydrogenase gene (*ALDH1A2*)) does not improve the value of a risk-stratification tool they previously developed [51]. Of note is that OAC with LOH mutations of both 17p and 18q had worse survival than cancers with no or one allelic loss [52]. Assessing all genetic and environmental risk factors together is therefore more prudent than examining any one individual risk factor.

2. Barrett's oesophagus (BO)

2.1. Epidemiology, histology and diagnosis

Barrett's oesophagus (BO) is the top-ranking risk factor for the development of OAC [6]. In this condition, normal squamous oesophageal epithelia are transformed into columnar cells and goblet (mucous-secreting) cells [6]. Researchers have not been able to link BO to every OAC case, partly due to the lack of screening and limited surveillance of BO, or due to the possibility that not all OAC cases develop from BO [46]. The adult prevalence of BO in the Western world is 0.5%–2.0% [53]. The

pooled, annual incidence (number of new cases) of OAC from NDBO patients ranges from 0.3% to 0.6% whereas the pooled, combined annual incidence for HGD and OAC was between 0.9% and 1.0% [54]. Patients with LGD are more likely to develop OAC, compared to patients with NDBO [55]; there is thus a need to implement a specific BO strategy for this group [55]. The average annual BO incidence rates have risen by 159% from 1993-1997 to 2002-2005 [56]. Those with BO have a 40-50-fold higher risk of developing OAC, compared to the general population [6]. The study of BO is valuable as rarely does a cancer have a pre-condition to detect early warning signs.

The diagnosis of BO requires correlation of findings from both upper endoscopy and histopathology [57]. The distal oesophagus normally is covered with squamous mucosa that is white or pale pink in colour [57]. With repeated inflammatory stimuli such as reflux, the squamous mucosa can undergo a metaplastic process and become salmon-coloured mucosa on endoscopy [57]. Histologically, the squamous epithelial lining of the oesophagus is replaced by simple columnar epithelia (resembling those of the colon, small bowel or stomach) alongside goblet (mucin-secreting) cells [57].

By current definition, BO is the presence of longer than 1cm, salmon-coloured columnar mucosa above the gastroesophageal junction endoscopically; this mucosa has to be proven to harbour intestinal metaplasia on histology [57]. The term long-segment BO is defined as the length of columnar mucosa > 3cm, and short-segment BO is used when the length is between 1 and 3 cm [57]. Increased length of columnar mucosa is associated with about a 46% increase in the risk of cancer progression for every 1cm increase in the BO length [57].

2.2. Origin of BO cells

The origin of BO cells (be there one or multiple sources) is currently unclear. It is believed that the transformation of squamous oesophageal cells to BO tissue

follows the concept of clonal expansion; here, an originating cell expands into a clonal population retaining early mutations, and later develop mutations that may expand into new subclonal populations [46]. A phenotypically altered cell, regardless of its tissue origin, is selected for owing to the acidic environment created by reflux within the oesophagus [46]. This cell has an advantage over the squamous epithelia surrounding it, and can expand clonally. [46]. Among the sources of origin of BO cells cited are the gastric cardia; oesophageal submucosal glands; oesophageal basal cells; oesophageal stem cells; or bone marrow progenitor cells [46], [58].

2.3. Genetic disposition and environmental risk factors for BO

A small number of BO cases may be heritable [44], [53]. A review by Van Nistelrooij et al. noted that approximately 6-7% of BO cases are considered familial (i.e. two or more relatives having a diagnosis) [44] and treatment outcomes between familial and non-familial cases may vary [44]. Ek et al. noted a that a predisposing genetic background (many germline genetic variants of small effect) accounted for 35% of BO heritability [43] and that BO and OAC share many polygenic (multiple-gene) effects [43]. Similar to OAC, BO specimens contained LOH mutations in 17p (14% of specimens) and in 18q (32% of cases) [52]. The literature has highlighted a minor role for *TP53*, *SMAD4* and *MUC5AC* in BO, more so in dysplastic cases [49], [59], [60]. In a retrospective analysis of BO tissue, Stachler et al. observed higher numbers of *TP53* mutations in BO patients who later progressed to HGD or OAC [59]; profiling patients based on *TP53* status may help in identifying the most at-risk patients. A loss of the SMAD4 protein promotes tumorigenesis in dysplastic (HGD) BO [60]. Finally, Arul et al. noted that BO tissue strongly expressed *MUC5AC* in the superficial columnar epithelium [49]; combined with the reported LOH mutation in OAC [47], the observation by Arul et al. suggests that *MUC5AC* expression is tumour protective in BO.

BO-related risk factors are consistent with OAC ones: nine meta-analyses have demonstrated (at least once) that BO is more common in males than females; in

subjects who have ever smoked (ever smoked at least one cigarette in their lifetime); in subjects with obesity; in subjects with prolonged symptoms of GORD; in subjects who have never had a *H. pylori* infection; and in subjects with hiatus hernia (the displacement of part of the stomach to the chest area) [61].

2.4. The role of extracellular acid (EA) in the development of BO and OAC

GORD is among the most notable of BO risk factors [33], [62]. The condition is highly prevalent (10% - 20% of the general population) in Europe and three to six percent of individuals with reflux-predominant symptoms may have BO [63], [64]. Acid and BA reflux from the stomach (the physiological mechanism underlying GORD) has been associated with the development of OAC [33]. Cancer tumours sometimes have acidic extracellular environments due to the Warburg effect (enhanced anaerobic metabolism) and to hypoxia within tumours [65], [66]. Cancer cell-lines (prostate, colon, pancreatic, lung and breast cancers) exposed to acidic environments (pH ranged from 6.0 to 6.8, depending on the study) have been to have increased markers of cancer development, such as ROS production [67], [68], [69], [70], [71], [72]. Other cancer cell lines (prostate, lung and melanoma cancers) have exhibited metastasis-related behaviour (such as epithelial mesenchymal transition) upon exposure to acidic environments (pH ranged from 5.9-6.8, depending on the study) [73], [74], [75]. Investigating the mechanisms underlying these associations would provide valuable insights relating to cancer formation, including OAC formation.

2.5. Gender disparities in BO and OAC

Data from European and North American populations indicate that OAC is nine times more common in men than in women [76]. In Ireland, the 2009, male, age-standardised incidence rate (ASR) for OAC was 7.70 cases per 100,000 individuals, whereas the corresponding female ASR was 1.65 per 100,000 individuals [77]. In 2012, the age-standardised incidence rate for OAC in England was 17 per 100,000 for men and 3.8 per 100,000 for women [3].

The nine meta-analyses compiled by Gatenby and Soon identified one meta-analysis on the topic of male gender as an increased risk factor for BO [61]. A meta-analysis of 32 BO studies by Cook et al. noted a higher pooled male:female ratio in the prevalence of BO across the 32 studies [78]. Other BO studies [79], [80] were published in the same year. Ford et al. conducted a retrospective case-control analysis with a cross-sectional study to determine the risk of BO in a large, United-Kingdom population of 20,320 patients: these patients were 2.7 times more likely to be male [79]. A retrospective study was carried out by Falk et al. [80]. There were 839 patients in the registry (628 men and 211 women) [80]. The authors reported that: BO segment length was greater in men than in women; women were less likely to have prevalent high-grade dysplasia or cancer than men; and, during a mean follow-up of 4.72 years, there was an incidence rate of 1 in 179 patient-years of follow-up for women and 1 in 91 patient-years of follow-up in men [80]. The most recent systematic review and meta-analysis on the topic was carried out by Melquist et al. in 2022 [81]. Ten studies with 19,337 patients (50.6% women) reported on prevalence and six studies with 5,137 patients reported on neoplastic progression of disease between genders [81]. The analysis demonstrated a 70% lower rate of prevalence and a 60% lower rate of neoplastic progression of BO in women [81]. In line with all other observations, histologically confirmed BO frequency varied not only by gender but also by ethnicity, with non-Hispanic white males having the highest frequency and African American females having the lowest [82]. The underlying reason for this male:female disparity in both OAC and BO remains unknown [83]. One theory is that men lose the Y-chromosome in certain cells (including BO and OAC cells) as they age [84], [85]. Whether loss of the Y-chromosome contributes to the overall instability of BO and OAC cells has yet to be comprehensively studied.

2.6. Limited surveillance of BO

Screening for BO in the general population or in patients with GORD is currently not in place in either Ireland or the UK [86], [87]. Compared to the financial burden of treating and managing OAC, surveillance for BO and OAC would be a cost-effective method to reduce the burden of the disease and the precursor [57]. Endoscopic

monitoring with histopathological assessment of dysplasia is the only current method of surveillance with sufficient evidence to be recommended [86]. Current UK practice is for endoscopy every 2 to 3 years for long-segment BO and 3-5 yearly for short-segment BO, with risk factors including smoking and family history determining precise intervals for individuals [88]. Surveillance regimens should take into account the presence of intestinal metaplasia and length of the BO segment [86]. Such surveillance is relatively effective in detecting OAC: OAC developed at an approximate rate of 6 per 100 patients-years in patients with BO, combined with HGD, in the first few years of follow-up [89]. Endoscopic imaging technologies are not without limitations [90]. Limited sensitivity and sampling bias causes many dysplastic lesions to be missed [90]. There is also low inter-observer reproducibility among pathologists in grading dysplasia, leading to overdiagnosis or underdiagnosis [90]. The establishment of BO biomarkers would be a useful supplementary BO diagnostic method.

3. Treatment options for OAC

3.1. Grading and staging of OAC

The treatment regimen chosen for a patient depends on the grade and stage of the OAC tumour in question [57]. Tumour grade refers to changes in the morphology of cells, as assessed by microscopy. The grade of OAC indicates the potential aggressiveness and behaviour of the tumour. Current grading methods for OAC are based on the degree of differentiation of the tumor cells. Well-differentiated tumours (low-grade) generally grow and spread more slowly than poorly differentiated or undifferentiated tumours (high-grade) [57]. Tumour grading systems usually categorise specimens in to three or four grades [91]. Among the grading systems in use are the WHO and AJCC systems [91]. These methods categorize tumours into four grades and guide treatment decisions [91]. The UK and Ireland generally follow the AJCC grading system [91], [92].

The metastatic stage refers to tumor location and whether there was any metastasis in the lymph nodes or in distant sites [91], [93]. OAC staging usually follows the Tumour, Nodes and Metastasis (TNM) format: the size and extent of the

primary tumour is assessed; the presence and extent of regional lymph node involvement is observed; and the presence of distant metastasis is highlighted [91], [93]. Figure 1.1 details the T, N and M status and histological grade definitions for oesophagus and oesophagogastric junction cancer in the 7th edition of the AJCC [93].

T, N, and M status and histologic grade definitions for esophagus and esophagogastric junction cancer in the 7th edition of the American Joint Committee on Cancer (AJCC) Cancer Staging Manual

T status	
T_{is}	High-grade dysplasia
T1	Invasion into the lamina propria, muscularis mucosae, or submucosa
T2	Invasion into muscularis propria
T3	Invasion into adventitia
T4a	Invades resectable adjacent structures (pleura, pericardium, diaphragm)
T4b	Invades unresectable adjacent structures (aorta, vertebral body, trachea)
N status	
N0	No regional lymph node metastases
N1	1 to 2 positive regional lymph nodes
N2	3 to 6 positive regional lymph nodes
N3	7 or more positive regional lymph nodes
M status	
M0	No distant metastases
M1	Distant metastases
Histologic grade	
G1	Well differentiated
G2	Moderately differentiated
G3	Poorly differentiated
G4	Undifferentiated

Figure 1.1. T, N, M status and histologic grade definitions for oesophageal and oesophagogastric junction cancer in the 7th edition of the American Joint Committee on Cancer Staging Manual

Source: Berry MF. Esophageal cancer: staging system and guidelines for staging and treatment. J Thorac Dis. 2014 May;6 Suppl 3(Suppl 3):S289-97. doi: 10.3978/j.issn.2072-1439.2014.03.11. PMID: 24876933; PMCID: PMC4037413 [93].

3.2. The immune profile of BO and OAC

There is increasing evidence indicating that the immune profile of BO and OAC should be taken into account when choosing a treatment regimen [94], [95], [96], [97], [98], [99], [100], [101], [102], [103]. OAC is an obesity-associated cancer [102]; visceral obesity has been associated with immune cells infiltrating adipose tissue and a resulting, chronic, low-grade inflammation [104]. Left untreated, this inflammation-associated obesity (mediated by the production of pro-inflammatory cytokines) can lead to disease such as type 2 diabetes, non-alcoholic fatty liver disease and cancer [105]. The immune cell-profile of BO and OAC tissue switches from an anti-tumour profile to a pro-tumour one [104]. Kavanagh et al. observed that overall T-cell infiltration of oesophageal tissue is compromised in OAC [101], reducing the ability of the immune system to destroy cancer cells. Melo et al. demonstrated that gamma-delta T-cells are increased in the omentum and liver of OAC patients and are predominantly pro-inflammatory in these tissues [98]. It is known that natural killer cell dysfunction is associated with poorer patient outcome [96]. A cisplatin-resistant OAC cell-line expressed fewer activating natural killer cell receptor ligands than the cisplatin-sensitive version of the cell-line [95], highlighting the need for targeted therapies. Some anti-tumour cells (mucosal associated invariant T (MAIT) cells) persist in OAC tumours [100]. OAC cell-line viability was reduced upon exposure to expanded MAIT cells. Research has also illustrated a role for acidic environments in modulating immune function [94], [103]. Tumour acidosis contributes to cancer progression by promoting inflammation and inhibiting anti-tumour immunity [94]. Pro-inflammatory T-cells have been implicated in the initiation of oesophagitis (often associated with acid reflux) and higher proportions of pro-tumour, T Helper 2 cells were present in BO tissue [103]. Potential therapies would include acid neutralisation or suppression in conjunction with the promotion of anti-tumour immune cells.

3.3. Current treatments for OAC

Treatment for OAC is multifaceted and tailored to the individual patient. It can include surgery, chemotherapy, radiation therapy, targeted therapies, immunotherapies, or a combination of these modalities [106]. The Chemotherapy for oesophageal cancer followed by surgery study (CROSS) regimen, for example, incorporates chemotherapy, radiation therapy and surgery [107]. Early-stage cancer may be curable with surgery, while advanced-stage cancer often requires a multimodal approach to control the disease and manage symptoms [108]. Palliative care plays a crucial role in maintaining quality of life for patients with advanced cancer [109].

Surgery is a primary treatment for early-stage OAC and can be curative in these cases [108]. The most common surgical procedure is an oesophagectomy, where a portion of the oesophagus is removed [108]. Types of oesophagectomy include trans-hiatal oesophagectomy (removal of the oesophagus through incisions in the neck and abdomen); trans-thoracic oesophagectomy (removal of the oesophagus through the chest); and minimally invasive oesophagectomy (the use of keyhole surgery with smaller incisions) [108]. There also exists endoscopic mucosal resection: this can be used to treat early OAC that is limited to the mucosa layer and has not spread to lymph nodes [110], [111].

Chemotherapy uses drugs to kill cancer cells or stop them from growing [112], [113]. It can be used before surgery (neoadjuvant chemotherapy), after surgery (adjuvant chemotherapy), or as the main treatment for advanced cancer [112], [113]. Currently, most OAC patients are treated with a combination of chemotherapies such as taxanes (paclitaxel, docetaxel), platinums (carboplatin, cisplatin), or pyrimidine analogues (5-fluorouracil (5-FU) and capecitabine) [114].

Radiation therapy uses high-energy beams to kill cancer cells [115]. It can be used alone, combined with chemotherapy (chemoradiation), or before or after surgery [115]. The most common type of radiation therapy is external beam radiation

therapy while brachytherapy (internal radiation delivered directly to the tumour site) is used less often [115].

Two main Ireland- and United-Kingdom-specific documents on OAC treatment and management have been published in recent years: the 2018, NICE guideline (NG83, "Oesophago-gastric cancer: assessment and management in adults"; updated in 2023) [116]; and the 2019, Irish Department of Health National Clinical Guideline No. 19 ("Diagnosis, staging and treatment of patients with oesophageal or oesophagogastric junction cancer") [92]. Table 1.3 summarizes the guidelines for treating and managing OAC published in these documents.

Table 1.3. Summary of the guidelines (Ireland and United Kingdom) on the treatment and management of oesophageal adenocarcinoma (OAC)

	NICE (United Kingdom) [116]	**NCCP (Ireland)** [92]
Early-stage OAC	Oesophagectomy	Endoscopic mucosal resection for Barrett's-related neoplasia and T1a OAC (limited to the mucosa or muscularis mucosae)
	Radiotherapy (alone or in combination with chemotherapy) for people with T1b OAC (invasion of the submucosa) and who are unfit for an oesophagectomy	Radiofrequency ablation for any residual cancer
Later-stage OAC	Consider two-field lymph node dissection during the oesophagectomy	Transthoracic oesophagectomy for patients with locally advanced OAC
	Neoadjuvant chemotherapy and adjuvant chemotherapy	Transhiatal oesophagectomy for patients with OAC of high operative risk.

	Chemoradiotherapy before surgery	

Recent years have also introduced targeted therapies and immunotherapies for OAC. Targeted therapies for OAC focus on inhibiting specific molecules involved in cancer growth and progression, such as HER2, VEGF and EGFR [50], [110]. Trastuzumab, a HER2 inhibitor, is a standard treatment for HER2-positive tumors, while ramucirumab, a VEGF inhibitor, is used in second-line therapy [50], [110]. Other targeted therapies, like EGFR inhibitors, have shown limited success, but ongoing research explores combinations with other therapies and immunotherapy [50], [110].

Immunotherapy aims to harness the patient's immune system to fight cancer [50], [110]. The most well-known immunotherapy for OAC is PD-L1 inhibition [50], [110]. PD-L1 is a protein on cancer cells that can bind to PD-L1 on T-cells, inhibiting the immune response [50], [110]. Immunotherapies, like PD-L1 inhibitors, are designed to block this interaction and unleash the body's immune cells to attack cancer cells [50], [110]. The PD-L1 inhibitors are man-made monoclonal antibodies of PD-L1, which target PD-L1 on cells and prevent the interaction between cancer cells and immune cells [50], [110]. This treatment approach effectively inhibits cancer-cell growth and spread while minimizing harm to healthy cells [110]. Before administering these medications, cancer cells are examined to determine if they possess the genes, proteins, or other factors that the targeted therapies are designed to address [110]. Table 1.4 summarises the currently available treatments for OAC.

Table 1.4. Summary of the main available treatments for oesophageal adenocarcinoma (OAC)					
Non-surgical	**Surgical**	**Chemotherapy regimens**	**Radiation regimens**	**Targeted therapies**	**Immunotherapies**
Radio-frequency ablation [92]	Endoscopic resection [92]	CF [117]	External beam radiation therapy [116]	Anti-HER2 targeted therapies [116]	Anti-PD-L1 monoclonal antibodies [118]
	Oesophagectomy [92]	Carboplatin and paclitaxel [119]	Brachytherapy [120]	Anti-VEGF targeted therapies [50]	
		CX [121]		Anti-EGFR targeted therapies [50]	
		ECF [122]			
		FLOT [123]			
		FOLFOX [124]			

CF = Cisplatin and fluorouracil; CX = Cisplatin and capecitabine; ECF = Epirubicin, cisplatin and fluorouracil; FLOT = Fluorouracil, leucovorin, oxaliplatin and docetaxel; FOLFOX = fluorouracil, oxaliplatin and leucovorin; CROSS = Chemoradiotherapy for oesophageal cancer followed by surgery study (carboplatin and paclitaxel with concurrent radiotherapy); HER2 = human epidermal growth factor receptor 2; VEGF = vascular endothelial growth factor; EGFR = epidermal growth factor receptor; PD-L1 = programmed death-ligand 1

For advanced OAC, palliative care focuses on relieving symptoms and improving quality of life [109]. This can include placing a stent to keep the oesophagus open and help with swallowing; photodynamic therapy (the use of light-sensitive drugs and laser light to kill cancer cells); and pain management [109].

Although oesophageal cancer (OSCC and OAC) initially responds well to treatment, most patients recur and eventually die from the disease; new therapeutic options are therefore urgently needed [114]. It is important to note that these chemotherapies are not used exclusively on OAC cells and that they target healthy cells as well as cancer cells, resulting in unwanted side effects [125]. More selective therapies are warranted.

3.4. Emerging treatments for OAC

Research is ongoing, and new treatments are continually being developed and tested in clinical trials. Such research includes new immunotherapies (such as chimeric antigen receptor T-cell therapy) [126]; targeting cancer-stem-cell-associated pathways (e.g. Wnt/b-catenin, Notch, Hedgehog or Hippo pathways) [127]; targeting cancer-stem-cell-associated miRNA expression profiles [128]; novel drug delivery systems [129]; epigenetic therapy (including the use of DNA methylation inhibitors and histone deacetylase inhibitors) [127]; and lifestyle interventions [130]. Novel drug delivery systems (delivering drugs directly to tumour cells), in particular, would reduce unwanted side-effects.

4. Ca^{2+} signalling

4.1. Introduction to Ca^{2+} signalling

Intracellular Ca^{2+} is a key second messenger and regulator of cell function [131]. In response to stimuli, Ca^{2+} channels or receptors on the plasma membrane (PM) induce changes in the levels of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$); such changes are sensed by Ca^{2+} effector proteins and result in a change in cellular physiology [132]. Multiple cellular processes are Ca^{2+}-sensitive, including cell growth, gene transcription, cell-cycle control, contraction, proliferation, fertilization, learning and memory, cell crosstalk (when one or more components of signal transduction pathways affect each other), membrane excitability, secretion, metabolism; vesicle trafficking, steroid synthesis; autophagy; and apoptosis [132], [133]. In response to both extracellular and intracellular cues, $[Ca^{2+}]_c$ can be increased by up to two orders of magnitude [134]. Such $[Ca^{2+}]_c$ transients regulate cell biology [131]. These increases in $[Ca^{2+}]_c$ can occur through several different mechanisms [134]. $[Ca^{2+}]_c$ can be increased through the gating of ion-channel proteins, located in the PM, or of intracellular organelles, allowing the influx or release of Ca^{2+} into the cytoplasm; other proteins remove Ca^{2+} from the cell, maintaining homeostasis [131], [132].

4.2. Ca^{2+} toolkit proteins

The collection of ion channels, receptors, pumps, exchangers and effector proteins involved in Ca^{2+} signalling is referred to as the Ca^{2+} Toolkit [132], see Figure 1.2. These proteins include: voltage-gated Ca^{2+} channels (VGCCs); acid-sensing ion channels (ASICs); transient receptor potential (TRP) cation channels; inositol 1,4,5-trisphosphate receptors (IP_3Rs); ryanodine receptors (RYRs); Orai and stromal interaction molecule (STIM) proteins; junctophilin (JPH) proteins; sarcoplasmic (SR) and endoplasmic reticulum (ER) Ca^{2+} ATPase (SERCA) pumps; secretory pathway Ca^{2+} ATPase (SPCA) pumps; Na^+-Ca^{2+} exchangers (NCXs); mitochondrial proteins; lysosomal proteins; and Ca^{2+} effector proteins (Ca^{2+}-sensors, enzymes, Ca^{2+} buffers and transcription factors) [131]. Table 1.5 summarises Ca^{2+} Toolkit proteins and their respective functions.

Table 1.5. Summary of Ca^{2+} Toolkit Proteins [133], [135], [136], [137]				
Ca^{2+} Toolkit Protein(s)	**Mechanism of action**	**Main Anatomical Locations**	**Main Biological roles**	**Number of subfamilies**
VGCCs	Gated by changes in membrane potential	Muscle and nervous tissue	- Contraction - Secretion - Regulation of gene expression - Synaptic expression - Repetitive firing of action potentials	Ten
ASICs	- Na^{+} and Ca^{2+} permeable channels - Gated by various ligands and stimuli - Some are gated by protons	Brain and peripheral nervous system tissue	- Fear behaviour - Learning - Proprioception - Pain sensation	Seven
TRPs	- Cation channels	- Sensory neurons	- Pain, temperature, taste and touch	Twenty-seven

	- Located on the PM - Gated by various ligands and stimuli - Relatively non-selective permeability to Na^{+}, Ca^{2+} and Mg^{2+}.	- Central nervous system	- Homeostasis	
IP_3Rs	These receptors (IP_3Rs) propagate Ca^{2+} signals in the ER upon being triggered by their binding to IP_3.	- Cerebellum - Muscle tissue	- Muscle contraction - Neuronal activity - Hormone secretion - Gene transcription - Cell proliferation - Embryonic development - Immune response	Three
RYRs	These receptors also release Ca^{2+} from the ER and SR into the cytoplasm	- Skeletal muscle - Cardiac muscle - The brain	- Muscle contraction and relaxation - Neurotransmission - Secretion	Three

			- Cerebral artery vasospasm	
Orai channels	SOCE	- Smooth muscle - Dendritic spines - Osteoblasts	- Immune-cell function - Cell growth and differentiation - Neurotransmitter release	Three
STIM proteins	SOCE	Ubiquitous expression	- Regulates ROS production and phagocytosis in neutrophils - Neuronal function - Cytokine production - Microtubule dynamics	Two
JPH proteins	- They form junctional membrane complexes; this is achieved by anchoring the SR or ER to the PM	- Skeletal muscle - Smooth muscle - Cardiac muscle - The brain and sensory neurons	- Muscle contraction	Four

	- These complexes allow the entry of extracellular Ca^{2+} into the ER upon Ca^{2+} depletion.			
SERCAs	Utilizes energy from ATP hydrolysis to transport Ca^{2+} ions from the cytoplasm to the SR / ER	Muscle tissue	- Muscle relaxation	Three
SPCAs	- SPCAs pump Ca^{2+} from the cytoplasm into the Golgi lumen - The transport Mn^{2+} into the Golgi	- Brain - Testes - Epididymal fat pads - Heart, liver, lung, kidney - GI system - Genito-urinary system - Nervous system	- Protein glycosylation - Cell migration during embryonic development - Metal homeostasis	Two

		- Mammary glands - Salivary glands - Colon epithelial cells		
NCXs	- Forward mode: they extrude Ca^{2+} from the cell (PM NCXs) - Reverse mode: they allow the influx of Ca^{2+} into the cell	- Heart - Neurons - Vascular smooth muscle - Kidneys - Skeletal muscle	- Muscle contraction and relaxation - Cellular homeostasis - Nerve cell signalling - Photoreceptor cell activity	Three
GPCRs	Mediate Ca^{2+} signalling through the activation of phospholipase C and the subsequent generation of second messengers like IP_3 and DAG.	- Neurons - Heart - Adipose tissue - Pancreas - Ovaries	- Neurotransmission - Regulating heart rate and contractility - Regulating fat metabolism - Controlling insulin and glucagon secretion	Four

			- Mediating steroid hormone signalling	
RTKs	Activation results in the release of Ca^{2+} from the ER	A wide range of tissues	- Cell proliferation - Cell differentiation - Cell migration - Gene transcription - Neuronal function - Tissue homeostasis	Twenty
Ca^{2+} sensors, enzymes and buffers	- Sensors: expose binding sties for downstream target proteins - Enzymes: act as biological catalysts upon binding with Ca^{2+} - Buffers: maintain $[Ca^{2+}]_c$ by regulating Ca^{2+} diffusion	- Neurons - Muscle - Epithelial tissue - Heart - Kidney - Intestine	- Muscle contraction - Nerve impulse transmission - Cell division - Hormone secretion	Wide range of subfamilies

Ca^{2+} transcription factors	- Ca^{2+} transcription factors bind to specific DNA sequences and either promote or inhibit the transcription of genes	- Muscle - Neurons - Vascular tissue	- Gene transcription or inhibition of gene transcription - Chromatin remodelling	Wide range of subfamilies

VGCCs = Voltage-gated Ca^{2+} channels; ASICs = Acid-sensing ion channels; TRPs = Transient receptor potential channels; PM = plasma membrane; IP_3Rs = Inositol trisphosphate receptors; IP_3 = Inositol trisphosphate; ER = endoplasmic reticulum; RYRs = Ryanodine receptors; SR = sarcoplasmic reticulum; SOCE = Store-operated Ca^{2+} entry; STIM proteins = Stromal interaction molecule proteins; ROS = Reactive oxygen species; JPH proteins = Junctophilin proteins; SERCAs = Sarco/Endoplasmic Reticulum Ca^{2+} ATPases; SPCAs = Secretory Pathway Ca^{2+} ATPases; Mn^{2+} = manganese; GI = gastro-intestinal system; NCXs = Na^+ / Ca^{2+} exchangers; GPCR = G-protein coupled receptor; DAG = diacylglycerol; RTK = receptor tyrosine kinase

Together, the Ca^{2+}-regulating and -sensing proteins within a cell can be considered a toolkit, which orchestrates stimulus-response coupling [132], see Figure 1.2. There are four categories of Ca^{2+} signalling components: Ca^{2+}-mobilising signals, triggered by various stimuli; “ON mechanisms” which feed Ca^{2+} into the cytoplasm; the actual signalling process; and the “OFF” mechanisms, which remove Ca^{2+} from the cytoplasm and restore the resting state [132], [134]

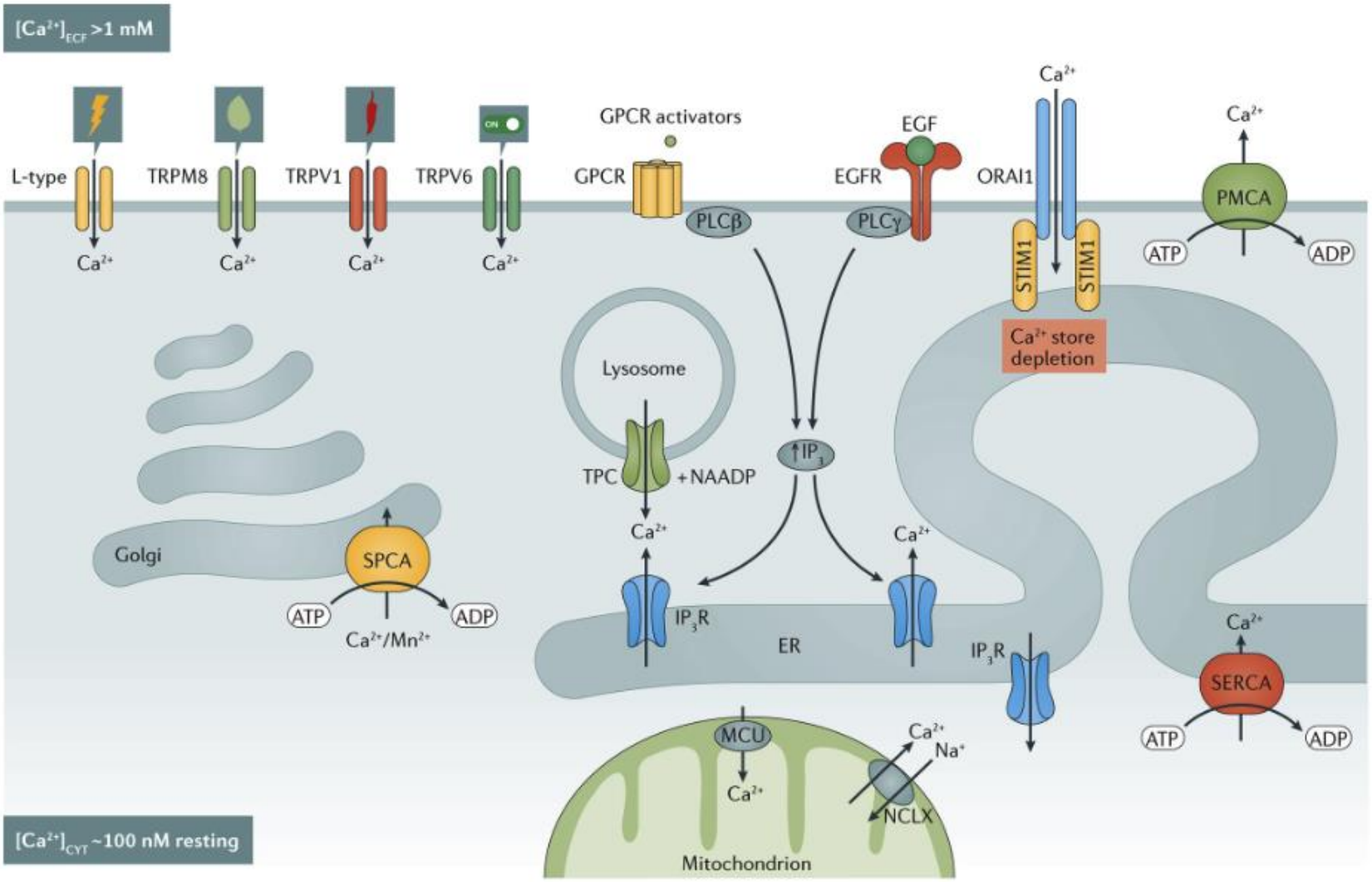
[Ca2+]ECF >1 mM
L-type
TRPM8
TRPV1
TRPV6
Ca2+
GPCR activators
GPCR
PLCβ
EGF
EGFR
PLCγ
ORAI1
STIM1
STIM1
Ca2+ store depletion
PMCA
ATP
ADP
Lysosome
TPC
+NAADP
IP3
Golgi
SPCA
ATP
ADP
Ca2+/Mn2+
IP3R
ER
IP3R
SERCA
MCU
Ca2+
Na+
NCLX
Mitochondrion
[Ca2+]CYT ~100 nM resting

Figure 1.2. Summary of the functions of Ca^{2+} Toolkit proteins in the cell

Various channels (voltage-gated Ca^{2+} channels (VGCCs), transient receptor potential (TRP) channels, acid-sensing ion channels (ASICs, not shown) and ORAI channels), receptors (G-protein coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs)) and pumps (plasma membrane Ca^{2+} ATPases (PMCAs)) lie on the plasma membrane (PM). In response to various stimuli, VGCCs, ASICs and TRP channels allow the influx of Ca^{2+} into the cell.

The activation of GPCRs and RTKs results in the production of inositol 1,4,5-trisphosphate (IP_3); IP_3 binds to IP_3 receptors (IP_3Rs) on the endoplasmic reticulum (ER) and release Ca^{2+} from the ER into the cytoplasm. Ryanodine receptors (not shown) also release Ca^{2+} from the ER into the cytoplasm.

When Ca^{2+} stores in the ER are depleted, ORAI channels (in combination with stromal interaction molecule (STIM) proteins) draw Ca^{2+} in from outside the cell into the ER. JPH proteins (not shown) contribute to $[Ca^{2+}]_c$ homeostasis by forming junctional membrane complexes (between the SR or ER to the PM). These complexes allow the entry of extracellular Ca^{2+} into the ER upon Ca^{2+} depletion. When $[Ca^{2+}]_c$ levels are too high, Ca^{2+} is pumped back into the extracellular space, the ER and the Golgi apparatus by PMCAs, sarcoplasmic / ER Ca^{2+} ATPases (SERCAs) and secretory pathway Ca^{2+} ATPases (SPCAs), respectively; SPCAs also pump Ca^{2+} back into the ER or SR.

The mitochondrial Ca^{2+} uniporter transports Ca^{2+} into the mitochondrion. Sodium-Ca^{2+} exchangers (NCXs) are found on the PM, the ER and the mitochondrion. When working in forward mode, they extrude Ca^{2+} from the cell (PM NCXs) whereas they allow the influx of Ca^{2+} into the cell when working in reverse mode. NCXs on the ER refill the ER with Ca^{2+} , whereas NCXs on the mitochondria release Ca^{2+} into the cytoplasm.

The lysosome also stores Ca^{2+} and releases it into the cytoplasm as needed. Among the Ca^{2+} toolkit proteins located on the lysosome include two-pore channels (TPCs), TRP channels, mucolipins and P2X4 ATP-activated cation channel.

Changes in $[Ca^{2+}]_c$ are detected by effector proteins, including Ca^{2+}-sensors, enzymes, Ca^{2+} buffers and transcription factors (not shown).

Source: Monteith, G., Prevarskaya, N. & Roberts-Thomson, S. The calcium–cancer signalling nexus. *Nat Rev Cancer* **17**, 373–380 (2017). https://doi.org/10.1038/nrc.2017.18 [137].

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4.3. The role of Ca^{2+} toolkit proteins in cancer development

Dysregulated Ca^{2+} signalling, and alterations in the Ca^{2+} toolkit, promote tumour-cell transformation, proliferation, migration and the evasion of apoptotic cell death [138]. Such changes in phenotype are achieved via a number of Ca^{2+} toolkit dependent mechanisms [133].

A review of the Oncomine clinical database [139] of nineteen cancer subtypes revealed that VGCCs were predominantly underexpressed, compared to non-cancerous controls [140]. High expression of a T-type channel, CACNA1G, has been reported to be associated with a reduction in proliferation and an increase in apoptosis in prostate cancer cells [140].

Several TRP family channels (including TRPA1, TRPC3, TRPC5, TRPC6, TRPM1, TRPM2, TRPM4, TRPM6, TRPM8, MCOLN1, MCOLN2, MCOLN3, TRPV1, TRPV2, TRPV3, TRPV4, TRPV5 and TRPV6) have been implicated in cancer development [141]. *TRPA1* activation has been implicated in prostate cancer (inducing angiogenesis in endothelial cells) and in breast and lung cancer (where the channel activates Ca^{2+}-dependent antiapoptotic pathways) [141]. TRPC3-mediated Ca^{2+} influx has been suggested as an endothelial cell attraction factor in prostate cancer, thus promoting angiogenesis [141]. TRPC3 overexpression has been detected in triple-negative breast cancer cells and plays a role in preserving proliferation and resistance to apoptosis [141]. TRPC5 was found to be overexpressed in colorectal cancer cells resistant to 5-FU chemotherapy [141]. TRPC6 has been found to be overexpressed in breast cancer tissue and its silencing results in a significant reduction in cell growth [141]. TRPM1 protein expression is

associated with tumor progression and survival in acral melanoma, supposedly because of the activation of Ca^{2+}/calmodulin-dependent protein kinase II [141]. TRPM2 is highly expressed in many human cancers (neuroblastoma, breast, gastric, lung, pancreatic, prostate cancer, squamous cell carcinoma, and T-cell leukaemia), where its activation increases malignant cell survival [141]. The modulation of TRPM2 via oxidative stress in several pathological conditions has been reported [141]. TRPM2 promotes the generation and release of matrix metalloproteinases by mediating Ca^{2+} influx and promoting extracellular matrix (ECM) degradation [142]. TRPM4 channel expression has been described in several cancers, including prostate, urinary bladder, cervical, colorectal, liver, and large B cell lymphoma [141]. In cancer cells, TRPM4 upregulation was associated with cancer cell migration, proliferation, and invasion [141]. The downregulation of the TRPM6 channel is present in 80% of primary tumours in colorectal cancer cells, whereas its high expression increases patient survival [141]. TRPM8 (both the protein and the gene, *TRPM8*) exhibits an increased expression in several cancer subtypes, including colon, breast, and prostate tumours, and it is considered to be a useful prognostic marker [141]. An increased MCOLN1 expression level has been correlated with poor clinical outcomes of pancreatic ductal adenocarcinoma patients, significantly lowering overall survival [141]. *MCOLN2* silencing decreases proliferation and cell viability in glioma cell lines [141]. Moreover, a role for MCOLN2 in prostate cancer has recently been reported [141]. An analysis of the Cancer Genome Atlas [143] revealed that the downregulation of *MCOLN3* is associated with a relatively better patient survival in several types of cancers, including adrenocortical, breast invasive, uterine corpus endometrial, kidney renal clear cell, and kidney papillary cell carcinomas, colon, lung, lung squamous cell, rectal, and stomach adenocarcinomas, pheochromocytoma, and paraganglioma, thymoma, and uterine carcinosarcoma. TRPV1 is functionally expressed in human OSCC cells; it has been reported that TRPV1 overactivation supports the proliferation and/or migration of OSCC cells [141]. TRPV2 is implicated in the signalling pathways that mediate cell survival, proliferation, and metastasis [141]. It can be overexpressed in cancerous cells and silencing or blocking can trigger apoptosis and cell cycle arrest [141]. An increased TRPV3 expression has been associated with cancer-cell proliferation, survival, and

invasion [141]. Inhibition of TRPV4 suppresses the metastasis of hepatocellular carcinoma [144]. Altered TRPV5 expression (both that of the protein and the gene, *TRPV5*) has been identified among the different renal-cell carcinoma histopathological subtypes [141]. The expression of the *TRPV6* gene is remarkably upregulated in several human malignancies, including prostate and breast cancer [141].

The *ORAI1* gene (involved in SOCE) is highly expressed in nearly 40 types of carcinomas [145]. The activation of ORAI1 typically triggers calcineurin activation and subsequent nuclear factor of activated T cells (NFAT) translocation to the nucleus, involved in cell proliferation, apoptosis resistance, angiogenesis, cell invasion, migration, and metastasis [145]. Previous studies in the human breast, cervical, and other cancer types have shown the functional significance of STIM/Orai-dependent Ca^{2+} signals in cancer development and progression [146]. STIM1 and STIM2 have been reported to play a role in breast cancer metastasis [147], [148] and colorectal metastasis (STIM2, both protein and gene (*STIM2*)) [149]. IP_3Rs control different hallmarks of cancer [150]. In particular, IP_3Rs impact cell death and survival by mediating Ca^{2+} release from the ER and subsequently affecting mitochondria-regulated processes, including apoptosis [150]. Type 3 IP_3Rs (IP_3R3s) have anti-apoptotic and proliferative role in colorectal-cancer cells [151]. The RYRs have also been implicated in cancer development [152]. In an analysis of The Cancer Genome Atlas (TCGA), patients with *RYR* mutations (any of the three isoforms) exhibited significantly higher tumour mutational burden than those without *RYR* mutations for most of the 33 cancer types examined [152]. In uterine serous cancer, high expression level of RYR1 (both the protein and the gene, *RYR1*) in the tumours is associated with advanced stage of the disease [153]. Upregulation of RYR1-dependent Ca^{2+} signalling contributes to the malignancy of high-grade, serous ovarian cancer [154]. Inhibition of RYR1 suppressed proliferation, migration and enhanced apoptosis of uterine serous cancer through Ca^{2+}-dependent activation [153].

Golgi Ca^{2+} Pump Secretory Pathway Calcium ATPase 1 (SPCA1) is a key regulator of insulin-like growth factor receptor (IGFR) processing in a breast cancer cell-line;

inhibition of SPCA1 inactivates IGFR and results in tumour progression [155]. Peretti et al. reported a novel association between SPCA2, Orai1 and the voltage-gated potassium channel subfamily H member 1 (Kv10.1) in breast-cancer cell survival [156].

Mounting evidence indicates that many SERCA pumps are down-regulated in cancer [157]. For instance, the SERCA1 isoform (both the protein and the gene, *SERCA1*) is decreased in a cisplatin-resistant epithelial ovarian cancer cell-line [157]. SERCA2B (both the protein and the gene, *SERCA2B*) expression is significantly reduced in small-cell lung cancer, thyroid cancer, oral squamous cell carcinoma and colon cancer [157].

The downstream proteins in the Ca^{2+} signalling cascade include Ca^{2+}-sensors, enzymes, buffers and transcription factors; as such, these proteins also participate in cancer development. These proteins act in either a tumour-suppressive or tumour-promoting manner. Calbindin 1 protects osteosarcoma cells from apoptosis [158]. Following 5-FU treatment in colorectal cancer cell-lines, calbindin 2 is involved in apoptosis of these cells [159]. Loss-of-function calreticulin (*CALR*) mutations promote oncogenesis as they compromise natural and therapy-driven immunosurveillance [160]. Similarly, calnexin impairs the antitumor immunity of $CD4^+$ and $CD8^+$ T Cells [161]. The protein may be a novel sero-diagnostic marker for lung cancer [162]. Binding to Ca^{2+} activates the affinity of calmodulin for targets, such as Ca^{2+}-calmodulin-dependent serine-threonine kinase (CAMK) family [163]; dysregulation of these kinases in tumor microenvironment has been associated with immune-suppressive status. Ca^{2+} binds to calmodulin, which in turn activates the calmodulin-dependent phosphatase, calcineurin, to dephosphorylate the NFAT proteins, leading to their nuclear translocation and the induction of NFAT-mediated gene transcription [164]; dysregulation of this pathway can lead to cancer formation [165], [166]. c-Fos expression is a molecular predictor of progression and survival in epithelial ovarian carcinoma [167]. The presence of the protein inhibited cell proliferation, migration, and invasion, and promoted the apoptosis of osteosarcoma cells [168]. c-Fos also induced chondrogenic tumor formation in immortalized human mesenchymal progenitor cells [169] .

4.4. The role of EA and acid-sensing Ca^{2+} toolkit proteins in BO and OAC

Decreased extracellular pH has been implicated in the proliferation of some cancer-derived cell-lines (prostate, colon, lung, and breast cancers; pH from 6.0–6.8) [67], [68], [69], [70], [71], [72]. EA has also been implicated in cancer metastasis in other cell lines (prostate, lung, and murine melanoma cancers; pH ranged from 5.9–6.8) [73], [74], [170]. Proposed oncogenic mechanisms include the production of reactive oxygen species (ROS), increased genomic instability, increased proliferation, dysregulation of apoptosis, and increased inflammation [67], [68], [69], [70], [71], [72]. Di Pompo et al. demonstrated that the acidic environment of surrounding connective tissue may also contribute to pain in patients with breast-cancer bone metastasis [171]. Among the Ca^{2+} toolkit proteins implicated in acid-stimulated oncogenesis in these studies include: carbonic anhydrase 9 [69], G-protein coupled receptor 4 [71], sodium-proton exchangers [70] and ASICs 1-3 [67].

To date, research on alterations in the Ca^{2+} toolkit in BO and OAC, and the potential effect on patient survival, has been limited. Si et al. 2007 noted (in neutral-pH environments) that BO cells overexpressing the Ca^{2+} dependent signalling protein, NOX5-S, significantly increased inflammatory and DNA damaging markers [172]; such mechanisms are generally associated with oncogenesis [172]. Most studies on the topic have an acidic pH focus [173], [174], [175], [176], [177]. There has been one EA-related BO study on Ca^{2+} signalling carried out to date [173]. Hong et al. illustrated a pathway where an acidic environment activated NOX5-S expression, which in turn activated DNA methyltransferase 1 expression and increased promoter methylation of the p16 gene [173]; mutations (LOH on chromosomal arm, 9p) of the p16 gene (cyclin dependent kinase inhibitor 2A (*CDKN2A*)) are associated with the progression of NDBO to LGD [46].

The OAC-related studies focus on the interaction between EA, Ca^{2+} signalling and OAC development, as opposed to Ca^{2+} dependent mechanisms independent of EA

exposure [174], [175], [176], [177]. EA-exposure (as low as pH 5) increased intracellular Ca^{2+} levels in both OAC (FLO-1 and SEG1) and mouse metastatic melanoma cell-lines [174], [175], [176], [177], illustrating the potential for intracellular Ca^{2+} to be the mechanism by which EA modifies cancer cell biology. Kato et al. showed EA signalling triggered Ca^{2+} influx and activated phospholipase D in the murine metastatic melanoma cell-line [174]. Fu et al. illustrated that, in OAC, EA exposure increased intracellular Ca^{2+} which subsequently activated NOX5-S and CREB, which in turn increased proliferation and decreased apoptosis [175]. Zhou et al. showed that EA exposure resulted in NOX5-S-dependent PGE2 production and subsequent cell proliferation in OAC [176]. Li and Cao additionally showed that the pathway illustrated by Zhou et al. was mediated by Ca^{2+} signalling, which subsequently elevated reactive oxygen species and caused DNA damage [177].

Studies have reported proposed molecular mechanisms for EA exposure in GORD, BO and OAC [178], [179], [180]. Oxidative stress, caused by reactive oxygen species (ROS), leads to DNA damage, RNA damage, activation of oncogenes, and inhibition of tumor-suppressor proteins located in the oesophageal mucosa in GORD patients [178]. Exposure to EA has been linked to oxidative stress in BO [180]. Inflammatory mediators (ROS, interleukin-1 (IL-1), IL-6, IL-8, and transforming growth factor-beta (TGF-β)), located in the oesophageal mucosa in GORD patients, have also been implicated in carcinogenesis [179]. IL-1, IL-6, and IL-8 enhance epithelial turnover (OAC is epithelia-derived cancer) [179]. TGF-β is generally anti-inflammatory [179]. TGF-β-responsiveness is reduced in OAC due to alterations in its signalling pathway [179]. Altered Ca^{2+} signalling could be contributing to all of these candidate mechanisms.

Multiple Ca^{2+}-toolkit-related mechanisms link EA to increases in $[Ca^{2+}]_c$ and consequent changes in cell physiology [131], [132], [181], [182], [183], [184], [185], [186]. These include TRP channels (TRPA1, TRPC4, TRPM2, TRPM5, TRPV1, TRPV4 and TRPV5); GPRs, linked to PLC-activation and Ca^{2+}-release via IP_3Rs (GPR4, GPR65, GPR68 and GPR132); ASICs 1a, 1b, 2a and 3; vacuolar ATPases [ATPase

H^+ transporting V0 subunit A1 (ATP6V0A1), ATP6V0A2, ATP6V0A4, ATP6V0B and ATP6V0C]; proton exchangers (encoded by Solute Carrier 9A (SLC9A)1-9); solute-carrier family 4 member A (SLC4A; SLC4A1-5 and SLC4A7-11); chloride-bicarbonate exchangers [solute-carrier family 26 member (SLC26; SLC26A and SLC26A1-10)]; carbonic anhydrases (CA; CA1-4 and CA6-14); and the hydrogen voltage-gated channel 1 (HVCN1) [131], [132], [181], [182], [183], [184], [185], [186]. Despite the potential impact of these acid-sensing proteins, the roles of these proteins in the aetiology of BO and OAC are currently unknown.

5. Bile acids (BAs)

5.1. Introduction to BAs and their role in human physiology

Bile acids (BAs) are cholesterol derivatives which exert both metabolic and hormonal effects on the human body [187]. They are a component of bile whose functions include emulsification of lipids (to aid absorption in the small intestine), absorption of fat-soluble vitamins, metabolism and excretion of cholesterol, excretion of bilirubin and the dissolution of gallstones [187]. There are both primary (synthesized in the liver) and secondary (synthesized in the small intestine) BAs [187]. The primary BA, cholic acid (CA), is converted to either deoxycholic acid (DCA) or ursodeoxycholic acid (UDCA); whereas chenodeoxycholic acid (CDCA, the other primary BA) is converted to lithocholic acid (LCA) or UDCA [187]. These conversions are executed through the action of bacterial dehydroxylase enzymes in the small intestine [187]. BAs exist in both unconjugated forms or types that are conjugated to the amino acids, glycine or taurine (see Figure 3.1 of Chapter 3 for details on BA chemical structure) [188]. Conjugated BAs are the most predominant type in bile, accounting for greater than 90% of total BAs in the bile mixture of healthy individuals [188].

Bile is produced in the liver, stored in the gallbladder and released into the small intestine in response to ingested dietary fat [189]. Ninety-five percent of the BAs which enter the intestine are recirculated back to the liver and recycled, in a process known as enterohepatic recirculation [189]. Hydrophobic BAs, including glycocholic acid (GCA), LCA and DCA, are considered to be the most toxic of BAs, given that they are a major factor in inducing liver-cell death [190]. The toxic effects of BAs vary

depending on the pH of their environment, the concentration of the BA in question and the duration of BA exposure to cells [188], [191].

5.2. Role of BA exposure in cancers

Figure 1.3 summarizes the role of BAs in the development and prevention of the most frequently studied cancers on the topic to date [192]. Of key note is that UDCA exposure is consistently tumour protective, irrespective of cancer type [192]. Again, BA effects depended on the concentration of the BA in question (some studies use supraphysiological concentrations), the duration of exposure of the BAs to the tissue in question and the pH of the tumour microenvironment [192].

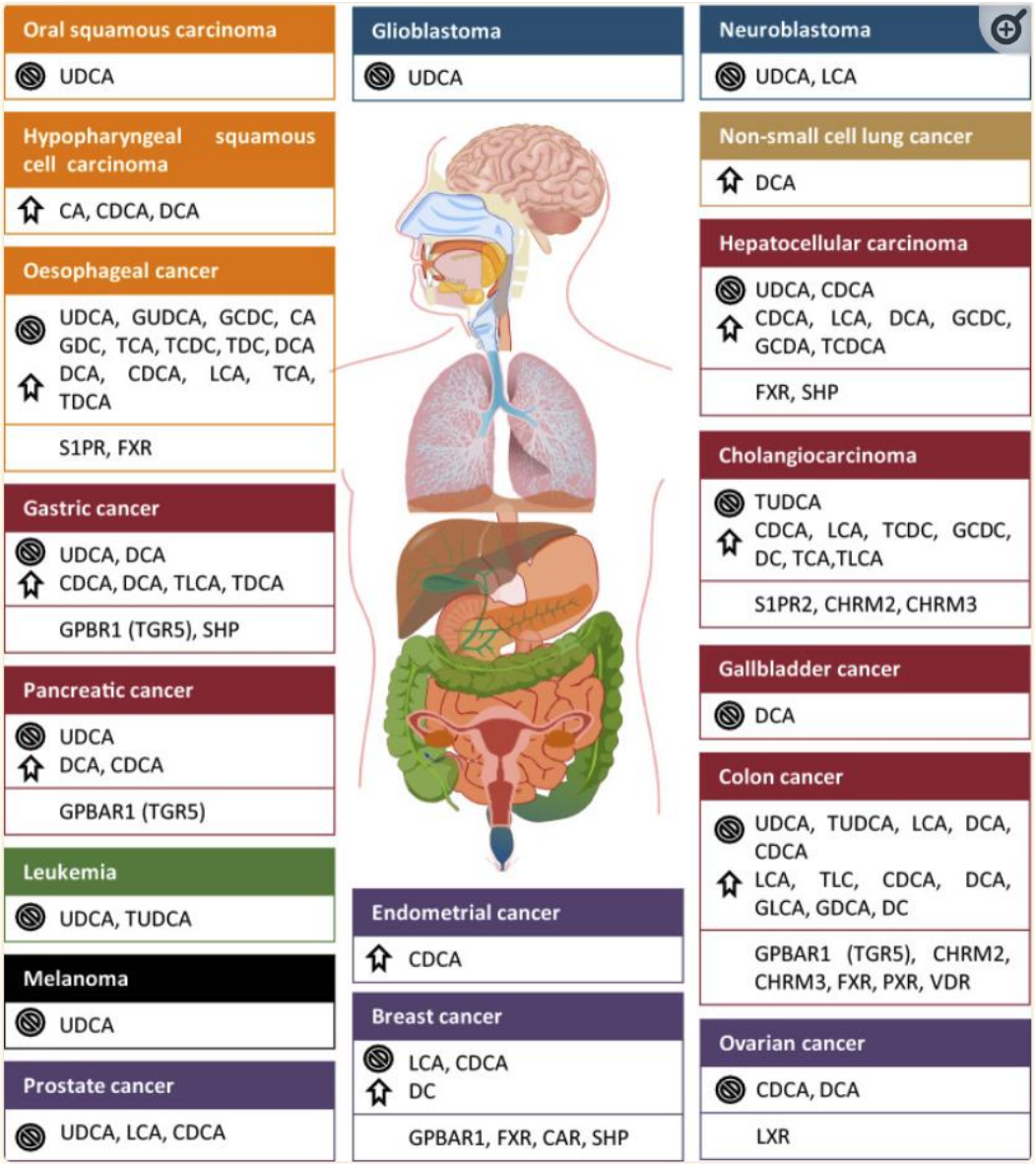

Figure 1.3. Roles of BAs and BA receptors in a variety of cancers

Some bile acids have opposing effects, depending on the cancer cell-line, the bile acid concentration, the bile acid exposure duration and other treatment conditions. The crossed circle symbol marks the tumour-suppressive associations and the arrow marks the tumour-promoting associations

CA = Cholic acid; CAR = Constitutive androstane receptor; CDCA = Chenodeoxycholic acid; CHRM2/M3 = Muscarinic receptor 2 and 3; DC = Deoxycholate; DCA = Deoxycholic acid; FXR = Farnesoid X receptor; GCDCA = Glycochenodeoxycholate acid; GCDC = Glycochenodeoxycholate; GDC = Glycodeoxycholate; GDCA = Glycodeoxycholic acid; GLCA = Glycolithocholic acid; GUDCA = Glycoursodeoxycholic acid; LCA = Lithocholic acid; PXR = Pregnane X receptor; S1PR2 = Sphingosine-1-phosphate receptor 2; SHP = Small heterodimer partner; TCA = Taurocholic acid; TCDC = Taurochenodeoxycholate; TCDCA = Taurochenodeoxycholic acid; TDC = Taurodeoxycholate; TDCA = Taurodeoxycholic acid; TGR5/GPBAR1 = G protein-coupled bile acid receptor 1; TLC = Taurolithocholate; TLCA = Taurolithocholic acid; TUDCA = Tauroursodeoxycholic acid; UDCA = Ursodeoxycholic acid; VDR = Vitamin D receptor.

Source: Režen T, Rozman D, Kovács T, Kovács P, Sipos A, Bai P, Mikó E. The role of bile acids in carcinogenesis. Cell Mol Life Sci. 2022 Apr 16;79(5):243. doi: 10.1007/s00018-022-04278-2. PMID: 35429253; PMCID: PMC9013344 [192].

It is thought that tumour-promoting BA exposure results in cancer-cell biology mechanisms such as ROS production, DNA damage, inflammation, cancer-cell

proliferation, apoptosis, apoptotic resistance and angiogenesis [193], [194], [195], [196], [197], [198].

5.3. Role of BA exposure in BO and OAC

GORD is a key risk factor for the development of BO and OAC and the refluxed contents from the stomach contain both acid and BAs [33]. Conjugated BAs, mainly taurocholic acid (TCA) and GCA, are the main BA constituents in GORD refluxate [199]. The secondary BA, DCA, and the conjugated BA, taurodeoxycholic acid (TDCA), are the most common BAs in the BO refluxate [199]. Furthermore, conjugated BA levels in the refluxate from patients with advanced BO or OAC are significantly higher than from patients with benign BO [192].

Two systematic reviews (published in 2011 and 2012, respectively) have focused on the role of BA exposure and the development of OAC, BO and / or reflux oesophagitis [188], [191]. The 2011 systematic review noted that BA exposure may contribute to the development of reflux oesophagitis and BO [191]. The 2012 systematic review that BA exposure can induce BO characteristics. [188] There is thus a need for an update on the topic, with an expanded focus on the effects of BAs at a neutral pH and on OAC development.

5.4. The role of BA signalling receptors in cancers, BO and OAC

It has been proposed that differences in the response of cancers to BAs lie in the differential expression of BA receptors between cancers [192]; such receptors include farnesoid X receptor (FXR), Takeda G-protein receptor 5 (TGR5), vitamin D receptor (VDR), sphingosine-1-phosphate receptor 2 (S1PR2) and pregnane X receptor (PXR) [200], [201], [202], [203]. Each of these receptors has their own homeostatic functions, but also have been associated with certain cancers, OAC and BO [192]. Their affinity for individual BAs also differs [204], [205]. The biological switch of BA receptor function from being cancer-protective to cancer-promoting in the oesophagus most likely depends on BA concentration, the duration of BA exposure, the disease state of the oesophagus and the pH of the tissue microenvironment [188], [191].

The farnesoid X receptor (FXR) is a nuclear receptor involved in the regulation of BA synthesis, transport, and absorption [206]. FXR plays a tissue-specific role in suppressing BA synthesis and promoting BA enterohepatic circulation; it also plays a part in regulating the inflammatory response [206]. FXR overexpression is found to be tumour protective in liver, colon, breast and prostate cancer but tumour promoting in pancreatic and lung cancer [192]. FXR expression has been shown to be increased in GORD compared to normal oesophageal tissue [207], and to be overexpressed in BO compared to normal mucosa, oesophagitis and OAC [208]. The TGR5 is a G-protein coupled receptor [200], [209], [210], [211], [212]. This receptor regulates proliferation, inflammation, adhesion, migration, insulin release, muscle relaxation and cancer development [200], [209], [210], [211], [212]. It interacts with TDCA and induces NOX5-S expression in OAC; this in turn increases cell proliferation, reactive oxygen species (ROS) production and DNA damage [209]. The VDR is a nuclear receptor [202], [213], [214], [215]. VDR plays a role in the inhibition of BA synthesis via suppression of cytochrome P450 family 7 subfamily A member 1 (CYP7A1), thus protecting liver cells during cholestasis (slowing of bile flow) [215]. It has affinity for LCA and has a key role in activating a pathway that detoxifies LCA [213]. VDR plays an essential role in innate immunity, inflammation, and cancer [202], [213], [214], [215]. VDR polymorphisms are reported to influence the development of various types of cancers, such as breast, liver, prostate, brain, and colon [202]. VDR protein was highly expressed in OAC and precancerous lesions [202]. The S1PR2 is a G-protein-coupled receptor [203]. S1PR2 is a critical receptor for the development and progression of different types of cancers and, although its role is varies depending on the tissue, most data support an anti-tumour function [216]. Glycochenodeoxycholic acid (GCDCA) can trigger apoptosis in hepatocytes through activating S1PR2 [192]. PXR, also a member of nuclear receptors expressed in the liver and intestine [201], [217], [218], [219], [220]. Activation induces detoxification of BAs through induction of cytochrome P450 family 3 subfamily A (CYP3A) expression [201]. PXR also detoxifies and eliminates endobiotic and xenobiotic compounds [217]. PXR promotes cancer cell apoptosis, inhibits proliferation, and regulates cell cycle [218]. PXR is most robustly activated by free or

conjugated lithocholic acid (LCA) and deoxycholic acid (DCA) [192]. In BO patients, PXR expression is higher in HGD versus LGD [221], supporting evidence that PXR expression might be able to separate HGD from LGD and non-dysplasia cases [221].

6. Aim and objectives of the current work

The aim of the present work was to investigate the potential role of Ca^{2+} signalling and BAs in the development or prevention of BO and OAC. Such an investigation may lead to novel therapeutic avenues and lifestyle interventions.

I hypothesize that dysregulated Ca^{2+} signalling (mediated by Ca^{2+} signalling proteins) plays a role in the development of OAC. I also hypothesize that BA exposure, independent of acidic pH environments, contribute to the development and prevention of BO and OAC.

My over-arching research questions are as follows:

A. Is the expression of genes encoding Ca^{2+} signalling proteins associated with improved or worsened OAC patient survival?
B. Does exposure to BAs, independent of acidic pH environments, contribute to the development and prevention of BO and OAC?

The objectives of the present work were as follows:

A. To investigate whether the expression of genes encoding Ca^{2+} signalling proteins is associated with improved or worsened OAC patient survival (CHAPTER 2).
B. To investigate the role of BA exposure in the development or prevention of BO and OAC (CHAPTER 3).

CHAPTER TWO. Alterations in the Ca^{2+} toolkit in oesophageal adenocarcinoma: a transcriptomic analysis

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Author contributions

JJM provided the original conception and design of the study. ALC, JJM, AN, GD, and DSC contributed substantially to the extraction, analysis, interpretation, and presentation of the data. RF supervised the analysis for the OCCAMS dataset. AN and GD worked on data from OCCAMS and DC analyzed data from UALCAN. TROD and SLM provided critical insights on cancer biology. AC wrote the first draft of the manuscript; JJM, TROD, and SLM contributed to sections of the manuscript. AN provided key statistical input. AC, JJM, AN, GD, and DC contributed to tables and figures. JJM prepared Figure 2.1. All authors participated in the revision and read and approved the submitted version.

Conflicts of interest

The authors declare no competing financial interests or other conflicts of interest.

Ethical approval

As the work is a bioinformatics analysis article, ethical approval was not necessary on the UCC authors' part. Ethical approval was obtained during the development of both individual datasets and informed patient consent was obtained prior to the collection of tissues [79-81].

Consent to participate

Not applicable.

Consent to publication

Not applicable.

Availability of data and materials

All data used in this manuscript were obtained from either The Cancer Genome Atlas (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga), accessed via the UALCAN portal (http://ualcan.path.uab.edu/), or the Oesophageal-Cancer, Clinical and Molecular Stratification (OCCAMS) dataset (https://www.mrc-cu.cam.ac.uk/research/rebecca-fitzgerald/clinical-studies/occams).

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Alterations in the Ca^{2+} toolkit in oesophageal adenocarcinoma: a transcriptomic analysis

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1. Abstract

Aim: To investigate alterations in transcription of genes, encoding Ca^{2+} toolkit proteins, in oesophageal adenocarcinoma (OAC) and to assess associations between gene expression, tumor grade, nodal-metastatic stage, and patient survival.

Methods: The expression of 275 transcripts, encoding components of the Ca^{2+} toolkit, was analysed in two OAC datasets: the Cancer Genome Atlas (via the University of Alabama Cancer (UALCAN) portal) and the oesophageal-cancer, clinical, and molecular stratification (OCCAMS) dataset. Effects of differential expression of these genes on patient survival were determined using Kaplan-Meier log-rank tests. OAC grade- and metastatic-stage status was investigated for a subset of genes. Adjustment for the multiplicity of testing was made throughout.

Results: Of the 275 Ca^{2+}-toolkit genes analysed, 75 displayed consistent changes in expression between OAC and normal tissue in both datasets. The channel-encoding genes, N-methyl-D-aspartate receptor 2D (*GRIN2D*), transient receptor potential (TRP) ion channel classical or canonical 4 (*TRPC4*), and TRP ion channel melastatin 2 (*TRPM2*) demonstrated the greatest increase in expression in OAC in both datasets. Nine genes were consistently upregulated in both datasets and were also associated with improved survival outcomes. The 6 top-ranking genes for the weighted significance of altered expression and survival outcomes were selected for further analysis: voltage-gated Ca^{2+} channel subunit α 1D (*CACNA1D*), voltage-gated Ca^{2+} channel auxiliary subunit α2 ?4 (*CACNA2D4*), junctophilin 1 (*JPH1*), acid-sensing ion channel 4 (*ACCN4*), *TRPM5*, and secretory pathway Ca^{2+} ATPase 2 (*ATP2C2*). *CACNA1D*, *JPH1*, and *ATP2C2* were also upregulated in advanced OAC tumor grades and nodal-metastatic stages in both datasets.

Conclusions: This study has unveiled alterations of the Ca^{2+} toolkit in OAC, compared to normal tissue. Such Ca^{2+} signalling findings are consistent with those from studies on other cancers. Genes that were consistently upregulated in both datasets might represent useful markers for patient diagnosis. Genes that were consistently upregulated, and which were associated with improved survival, might be useful markers for patient outcome. These survival-associated genes may also represent targets for the development of novel chemotherapeutic agents.

Keywords

Oesophageal adenocarcinoma, Ca^{2+} toolkit, acid-sensing, voltage-gated Ca^{2+} channel subunits, junctophilin 1, acid-sensing ion channel 4, transient receptor potential ion channel melastatin 5, secretory pathway Ca^{2+} ATPase 2.

2. Introduction

Globally, oesophageal malignancies are the sixth-leading cause of cancer-related mortality [1]. The age-standardized 5-year net survival for oesophageal cancers (OCs, 2010-2014) was 21.9% for Ireland [2], 16.2% for the UK [2], and 20% for the United States [3]. Causes of poor prognosis include late diagnosis, incomplete resection of tumours, and resistance to chemotherapeutic and radio-therapeutic interventions [4]. Histologically, there are two major distinct forms of OC: oesophageal squamous cell carcinoma (OSCC), derived from epithelial cells, and oesophageal adenocarcinoma (OAC), arising from glandular cells [5]. OSCC is most prevalent in Southeast Africa and Asia [5]. By contrast, OAC is the most prevalent form in North America and Europe [5]. Risk factors for OAC include obesity, Barrett's oesophagus (BO, the replacement of normal, squamous epithelia with metaplastic columnar epithelia [6]), and gastroesophageal reflux disease (GORD) [1-7]. Two stomach-derived stimuli impacting oesophageal cells, because of GORD, are hydrochloric acid and bile acids (BAs) [8-9]. Little is known, however, about how oesophageal cells detect and respond to these stimuli [10].

Intracellular calcium (Ca^{2+}) is a key second messenger in the cell [11-13]. In response to both extracellular and intracellular cues, cytoplasmic free Ca^{2+} $[Ca^{2+}]_c$ can be increased by up to two orders of magnitude [14, 15]. Such $[Ca^{2+}]_c$ transients regulate almost every aspect of cell biology including cell motility, gene expression, and cell death [11-15]. These increases in $[Ca^{2+}]_c$ can occur through several different mechanisms [15, 16]. $[Ca^{2+}]_c$ can be increased through the gating of ion-channel proteins, located in the plasma membrane (PM) or intracellular organelles, allowing the influx or release of Ca^{2+} into the cytoplasm [11]. These channels include those gated by changes in membrane potential (voltage-gated Ca^{2+} channels (VGCCs)), by ligands, by second messengers, by multiple stimuli (such as TRP channels) or by the depletion of intracellular Ca^{2+} stores (store-operated Ca^{2+}-entry (SOCE) channels, including Orai channels, which are gated by interactions with stromal interaction molecule (STIM) proteins) [11, 14 15, 17]. Intracellular Ca^{2+} stores, such as the endoplasmic reticulum (ER) and sarcoplasmic reticulum (SR), act as reservoirs of Ca^{2+}; this Ca^{2+} is released via channels such as the inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs) and ryanodine receptors (RYRs) [11, 14, 15, 17]. Golgi, secretory pathway Ca^{2+} ATPase (SPCA) 1 and 2, and the SR/ER Ca^{2+}-ATPases (SERCAs 1-3) are pumps that actively accumulate Ca^{2+} into intracellular stores, thereby decreasing $[Ca^{2+}]_c$ [11, 14, 15, 17]. $[Ca^{2+}]_c$ is also buffered by mitochondria, whose metabolic activities and effects on cell death are influenced by this second messenger [18]. As such, Ca^{2+} signalling can be influenced by mitochondrial proteins like those comprising the permeability transition pore, the mitochondrial Ca^{2+} uniporter (MCU), and mitochondrial Ca^{2+} exchangers [19, 20]. Changes in $[Ca^{2+}]_c$ are detected by effector proteins, including Ca^{2+}-sensors, enzymes, transcription factors, and motor proteins [12, 13]. Together, the Ca^{2+}-regulating and -sensing proteins within a cell can be considered a “toolkit”, which orchestrates stimulus-response coupling, Figure 2.1.

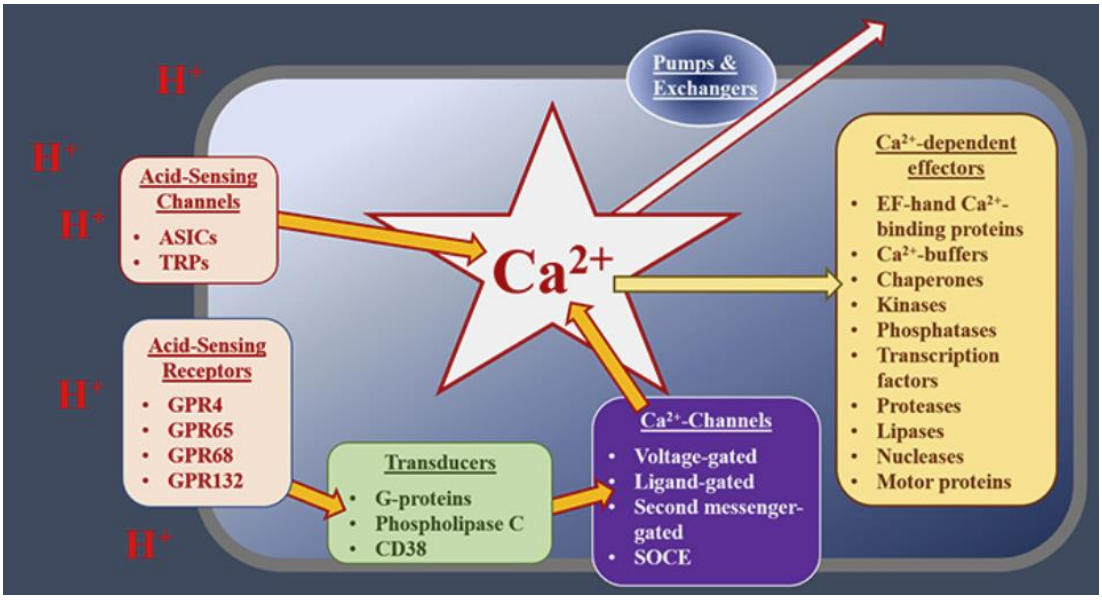


Figure 2.1. The potential role of the Ca^{2+} toolkit in OAC cells

At the cell surface, extracellular cues, such as decreases in extracellular pH (increased $[H^+]$), are detected by receptors or cation channels, which gate to allow Ca^{2+} influx. GPRs activate transducers, such as $G_{\alpha q/11}$, which stimulate phospholipase C (PLC) and subsequently increase the concentration of IP_3. This increased IP_3 concentration, in turn, gates IP_3Rs, releasing Ca^{2+} from the ER. Other Ca^{2+} channels and receptors respond to distinct stimuli or modify the signals generated by acid-sensing mechanisms. Elevated $[Ca^{2+}]_c$ alters the activities of various downstream effectors, resulting in the modification of cell physiology. Normal $[Ca^{2+}]_c$ is restored by buffers, pumps, and exchangers, operating in distinct subcellular compartments. Together, these receptors, Ca^{2+} channels, transducers, transporters, and effectors act as a Ca^{2+} toolkit, the components of which are distinctive for the type and condition of cells. ASICs: acid-sensing ion channels; TRPs: transient receptor potential ion channels; GPRs: G-protein coupled receptors; and CD38: cluster of differentiation 38.

Figure courtesy of Dr. John Mackrill.

Altered Ca^{2+} signalling can result in pathological or pre-pathological states [12-17]. In cancer cells, the Ca^{2+} toolkit is remodelled to enhance the hallmarks of malignancy. This remodelling can impact proliferation, metastasis, or the evasion of apoptotic cell death [21]. Remodelled mitochondrial proteins, in particular, play a role in cancer cell proliferation and the evasion of cell death [22-27]. Ca^{2+} signalling has been shown to

play critical roles in the biology of both normal and malignant oesophageal cells [21]. Extracellular Ca^{2+}, for example, promotes the proliferation of human esophageal epithelial cell line (HET-1A) cells.[28]. Alteration of the Ca^{2+} toolkit has been reported to contribute to the tumorigenic phenotype of other cancers, including OSCC [15, 17, 29-41]. Most studies to date have focused on the Ca^{2+} channel, TRP vanilloid 6 (TRPV6) [17, 34-40]. This channel was reported to be upregulated in breast, colon, ovary, prostate, and thyroid carcinomas [36]. In OSCC, however, TRPV6 was reported to be downregulated [35]. Increased *TRPV6*-expression has been associated with a better prognosis in cervical carcinoma [37]. The opposite effect (worsened survival with increased expression) has been reported in pancreatic cancer [38] and high-grade prostate cancer [34]. A sex-specific effect was noted in OSCC: male patients with *TRPV6*-downregulation had a poor 3-year disease-specific survival, whereas female counterparts showed enhanced survival [35]. Several TRPV6-related mechanisms have been proposed. *In vitro* analysis showed that *TRPV6*-silencing reduced breast cancer cell proliferation and promoted apoptosis [39]. In prostate-adenocarcinoma cells, TRPV6 supported high proliferation rates by providing constitutive Ca^{2+}-influx for subsequent downstream activation of the nuclear factor of activated T cells (NFAT) [40]. *TRPV6*-expression is promoted by the activation of vitamin D_3, oestrogen and androgen receptors [36]. Such an interaction explains, at least in part, the effect of TRPV6 on the hormone-related cancers of the breast and prostate, as well as the sex-specific association of TRPV6 with OSCC survival [36]. Finally, the Orai3 protein works in conjunction with TRPV6 to promote proliferation in prostate and breast cancer [17]. Some study has been carried out on the TRPV2 channel, a channel that belongs to the same family as TRPV6. Patients with OSCC, gastric cancer, triple-negative, breast cancer, and bladder cancer, who had high *TRPV2*-expression, displayed poorer survival [29-31, 41]. Similar to TRPV6 and TRPV2 (poor prognosis with high expression in numerous cancers), high expression of the PM Ca^{2+} ATPase 2 (PMCA2) conferred resistance to apoptosis and was associated with a poor prognosis [32]. Other Ca^{2+}-toolkit proteins implicated in various cancers include calcineurin, SERCA pumps, SPCAs, PMCAs, the IP_3R, RYRs, STIM proteins, T-Type VGCCs, TRPC1, TRPC3, TRPC6, TRPM2, TRPM7 and TRPM8 [15, 17, 34]. Some Ca^{2+}-permeable channels have been implicated in the enhanced migration of various

cancers: TRPC1, TRPM7, TRPM8, TRPV1, TRPV2, TRPV6, STIM1, Ca^{2+}-release activated Ca^{2+} modulator 1 (Orai1) and some types of VGCCs [17, 33]. It is not known whether there are Ca^{2+}-toolkit changes in OAC and if there is any association with tumor progression or patient survival.

Acidic conditions, occurring during GORD, are a key stimulus for the development of BO [6]; those with BO are 40-50 times more likely to develop OAC [42]. Decreased extracellular pH has been implicated in the proliferation of some cancer-derived cell lines (prostate, colon, lung, and breast cancers; pH ranged from 6.0-6.8) [43-48]. Extracellular acid (EA) has also been implicated in cancer metastasis in other cell lines (prostate, lung, and murine melanoma cancers; pH ranged from 5.9-6.8) [49-51]. Whether acidic extracellular environments result in oncogenesis in OAC, via the development of BO or by other mechanisms, remains unclear. Proposed oncogenic mechanisms include the production of reactive oxygen species (ROS), increased genomic instability, increased proliferation, dysregulation of apoptosis, and increased inflammation [43-56]**.** Exposure to EA has been linked to ROS production in BO [56]. Roesly *et al.* [52] showed that chronic exposure to BAs increased genomic instability and proliferation in BO and OAC cell lines. Indeed, the BA receptor (farnesoid X receptor (FXR)) is significantly overexpressed in BO (compared to normal mucosa, oesophagitis, and OAC) and may contribute to the regulation of apoptosis [53]. In nasopharyngeal carcinoma, BA-induced apoptosis (mediated by caspase-activated deoxyribonuclease) contributed to chromosomal rearrangements [54]. Inflammatory mediators (specifically: ROS, interleukin-1 (IL-1), IL-6, IL-8, and transforming growth factor-beta (TGF-β)), located in the oesophageal mucosa in GORD patients, have also been implicated in carcinogenesis [55]. Oxidative stress, caused by ROS, leads to DNA damage, RNA damage, activation of oncogenes, and inhibition of tumor-suppressor proteins [55]. IL-1, IL-6, and IL-8 enhance epithelial turnover (OAC is epithelia-derived cancer) [55]. TGF-β is generally anti-inflammatory [55]. TGF-β-responsiveness is reduced in OAC due to alterations in its signalling pathway [55]. Altered Ca^{2+} signalling could be contributing to all of these candidate mechanisms [57-69].

Multiple Ca^{2+}-toolkit-related mechanisms link increased extracellular $[H^+]$ to increases in $[Ca^{2+}]_c$ and consequent changes in cell physiology [10, 12]. These include TRP channels (TRP ankyrin 1 (TRPA1), TRPC4, TRPM2, TRPM5, TRPV1, TRPV4 and TRPV5); GPRs, linked to PLC-activation and Ca^{2+}-release via IP_3Rs (GPR4, GPR65, GPR68 and GPR132); ASICs 1-5; vacuolar ATPases (ATPase H^+ transporting V0 subunit A1 (ATP6V0A1), ATP6V0A2, ATP6V0A4, ATP6V0B and ATP6V0C); proton exchangers (encoded by Solute Carrier 9A (SLC9A)1-9); solute-carrier family 4 member A (SLC4A1-5 and SLC4A7-11); chloride-bicarbonate exchangers (solute-carrier family 26 member A (SLC26A1-10); carbonic anhydrases (CA1-4 and CA6-14); and the hydrogen voltage-gated channel 1 (HVCN1) [10, 12, 70-78]. A broad overview of the role of some of these Ca^{2+}-toolkit proteins in the cell has been detailed in Figure 2.1. Despite the potential impact of these acid-sensing proteins, their roles in the aetiology of BO and OAC are currently unknown.

A limited number of studies have evaluated alterations of the Ca^{2+} toolkit, and their potential association with patient outcome, in OAC. Even less research has investigated how certain Ca^{2+}-toolkit proteins may sense acidic environments and might, as a result, be remodelled to favour carcinogenesis. EA-exposure increased Ca^{2+}-levels in an OAC cell line (pH 5) and in a murine, metastatic, melanoma cell line (pH 5.4-6.5) [60, 61]. Li and Cao [60] showed that the EA-stimulated increase in $[Ca^{2+}]_c$ in their OAC cell line activated the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 5-S enzyme, which subsequently elevated ROS and caused DNA damage.

In the current study, we examined the transcript levels of various components of the Ca^{2+} toolkit in OAC samples and in normal oesophageal samples, using data from both The Cancer Genome Atlas (TCGA) and the oesophageal cancer clinical and molecular stratification (OCCAMS) consortium [79, 80]. We focused on a gene list of Ca^{2+}-toolkit components, updated from an original review of these [13], with the addition of known components involved in acid-sensing [10] and those related to mitochondrial function [19, 20, 22-27].

Specifically, we aimed to:

i) Interrogate two distinct transcriptomic datasets to assess whether there is differential expression of any of 275 Ca^{2+}-toolkit genes between OAC and normal tissue.

ii) Assess whether there is an association between the expression of Ca^{2+}-toolkit genes and patient survival in OAC.

iii) Examine whether these survival-associated genes are associated with tumor grade or metastasis.

3. Materials and methods

Two cancer transcriptome datasets were used in this study: TCGA (esophageal-carcinoma subset), accessed via the UALCAN portal [79, 81], and the OCCAMS dataset [80]. The TCGA dataset compared data from 89 OAC-tumor samples with 11 same-patient, normal-adjacent tissue (NAT) samples. The OCCAMS dataset compared data from 213 OAC samples with data from 15 normal-tissue samples (from independent OAC cases).

The complete list of genes examined, and their associated proteins, are detailed in Table S1. The expression levels of each of these 275 genes were assessed in OAC-tumor tissue and compared to expression levels in normal tissue. The mRNA expression levels of each gene were plotted as heat maps, using a log2 [transcripts per million (TPM)+1] scaled look-up-table. To determine which genes were most consistently and significantly altered between normal and tumor tissue across both datasets, a dataset-specific weighted rank (the probability of altered expression of each gene, compared to normal tissue, relative to other differentially-expressed genes) was calculated for each gene and the average of both dataset-specific ranks was then taken. The association of each gene with patient survival was investigated using Kaplan-Meier survival analysis. This analysis was carried out initially on all genes in the OCCAMS dataset only; any genes with a statistically significant effect on survival were subsequently analysed in UALCAN. A comparison was made between patients with high expression of the gene of interest (TPM reads above the upper

quartile) and those with low expression (TPM below the lower quartile). To determine which survival-associated genes were selected for further analysis, the extent to which they were differentially expressed across both datasets was evaluated. Again, an average of the two dataset-specific weighted ranks was computed for each gene. Genes having an association with survival, which also had the smallest, average, weighted ranks (1 being the smallest and 75 being the largest) for differential expression, were selected for further analysis.

Selected genes were also examined for differential expression across OAC tumor grades and nodal metastatic stages. Tumour grade refers to changes in the morphology of cells, as assessed by microscopy [82]. Histological tumor grades in this analysis were stratified based on cellular differentiation: Grade 1 was well-differentiated; Grade 2 was moderately differentiated, and Grade 3 was poorly differentiated. The metastatic stage refers to tumor location and whether there was any metastasis in the lymph nodes or in distant sites [82]. For the metastatic-stage boxplots, the following categories were used: N0 corresponded to the tissue having no regional lymph-node metastasis; N1 corresponded to the tissue having metastasis in 1 to 3 axillary lymph nodes; N2 corresponded to the tissue having metastasis in 4 to 9 axillary lymph nodes, and N3 corresponded to the tissue having metastasis in 10 or more axillary lymph nodes.

Statistical Analysis

Gene-expression data for tumor *versus* normal tissue were compared using Welch's *t*-tests. Associations of transcript-level (upper quartile *versus* lower quartile) with patient survival were presented in Kaplan-Meier plots and were statistically compared using log-rank tests. Associations between transcript levels and differentiation grade or lymph-node metastasis were presented as boxplots, compared by analysis of variance (ANOVA) with Tukey *post hoc* tests. For statistical comparisons, *P*-values of less than 0.05 were considered significant. An adjustment for multiplicity of testing (MOT) was made (for both UALCAN and OCCAMS data) using the false discovery rate (FDR) method [83, 84], for both the Welch's *t*-tests and

Kaplan-Meier, log-rank tests. Tukey, honest-significant-differences, *post hoc* tests served as an appropriate adjustment for MOT for UALCAN and OCCAMS ANOVA tests.

4. Results

Transcript levels of 275 genes, encoding components of the Ca^{2+} toolkit, were investigated in two OAC datasets. The full list of Ca^{2+} toolkit genes examined can be viewed in Supplementary Table S1. in the Appendices. A total of 392 statistical tests (Welch's *t*-tests, Kaplan-Meier, log-rank tests and ANOVA with Tukey *post hoc* tests) were carried out in the UALCAN portal and 638 in the OCCAMS dataset (data was not available for four genes in OCCAMS: G protein subunit gamma 7 (*GNG7*), mucolipin 1 (*MCOLN1*), junctophilin 3 (*JPH3*), and sorcin (*SRI*)). Initial survival analysis of 271 genes was carried out in the OCCAMS dataset (the dataset with the most statistical power). The resulting survival-associated genes significantly associated with survival were then analysed in UALCAN. One hundred and forty-eight gene variables [gene variable being defined as "a gene and an associated test", such as "voltage-gated Ca^{2+} channel auxiliary subunit α2 δ4 (*CACNA2D4*)-heat map" and "acid-sensing ion channel 4 (*ACCN4*)-survival"] were considered significant in the UALCAN portal. After adjustment for MOT, this number was reduced to 130 significant, gene variables. In the OCCAMS dataset, 233 gene variables were considered significant following adjustment for MOT.

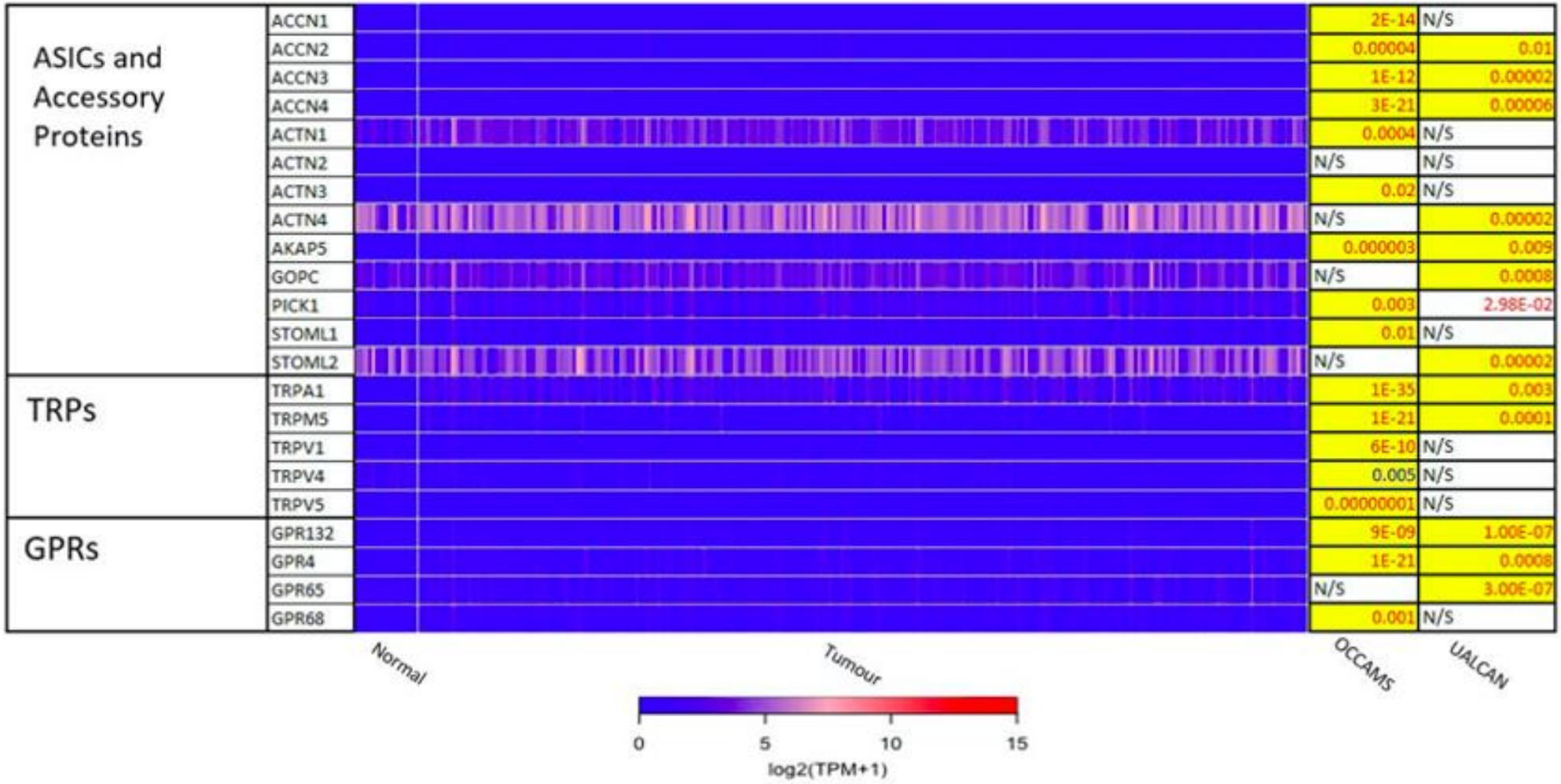
Expression analysis

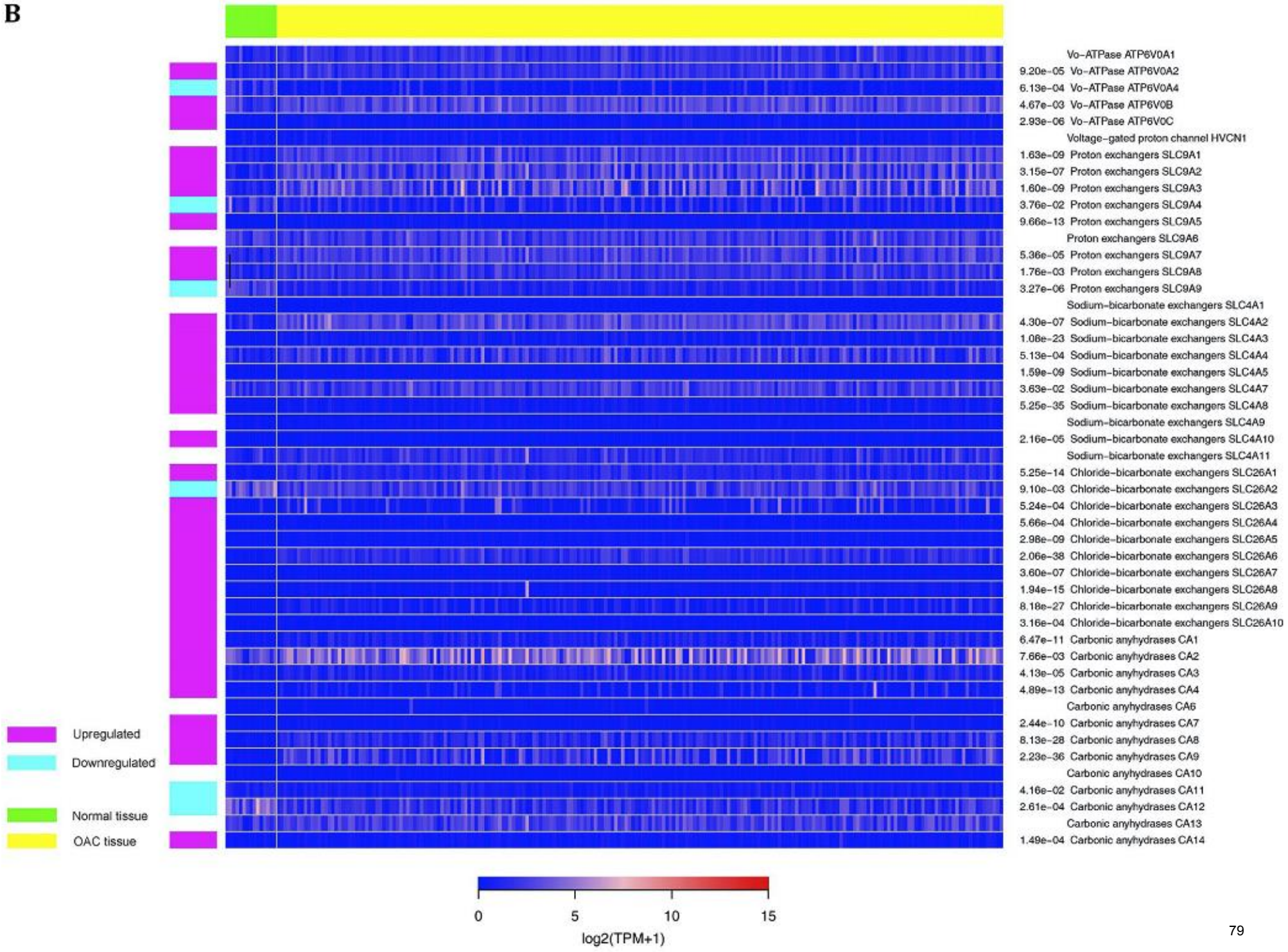
Of the 275 *t*-tests carried out on mRNA-expression levels in tumor *versus* normal tissue, 136 were statistically significant in UALCAN; after adjustment for MOT, this number was reduced to 118 (42.9% of the genes in the UALCAN dataset). Of the 271 heat-map-related *t*-tests carried out in OCCAMS, 182 (67.2%) were considered statistically significant following adjustment for MOT. Seventy-five of these genes (75/118 in UALCAN and the same 75/182 in OCCAMS) were differentially expressed across both datasets: 68 were upregulated compared to normal tissue; 4 (homer scaffolding protein 2 (*HOMER2*), *CACNA2D3*, voltage-gated Ca^{2+} channel, L-type, β 4 subunit (*CACNB4*), and *SLC9A4*) were downregulated; and 3 (*SLC26A9*, voltage-gated

Ca^{2+} channel auxiliary subunit gamma 4 (*CACNG4*), and two-pore segment channel 2 (*TPCN2*)) were differentially expressed in opposing directions in the two datasets.

The 271 genes presented in the heat-maps have been grouped into the following distinct functional categories: acid-sensing channels, receptors, and their accessory proteins; proton-regulating proteins (Figure 2.2A. and 2.2.B); Ca^{2+} pumps and exchangers (Figure 2.3); Ca^{2+} channels (Figure 2.4); Ca^{2+}-release channels and their accessory proteins (Figure 2.5); transducers (Supplemental figure S1); mitochondrial-associated, Ca^{2+}-toolkit genes (Supplemental figure S2); cytosolic Ca^{2+}-sensors and -buffers (Supplemental figure S3); Ca^{2+}-dependent chaperones (Supplemental figure S4); and Ca^{2+}-dependent effectors (Supplemental figure S5).

A


ASICs and Accessory Proteins
ACCN1
ACCN2
ACCN3
ACCN4
ACTN1
ACTN2
ACTN3
ACTN4
AKAP5
GOPC
PICK1
STOML1
STOML2
TRPs
TRPA1
TRPM5
TRPV1
TRPV4
TRPV5
GPRs
GPR132
GPR4
GPR65
GPR68
2E-14 N/S
0.00004 0.01
1E-12 0.00002
3E-21 0.00006
0.0004 N/S
N/S N/S
0.02 N/S
N/S 0.00002
0.000003 0.009
N/S 0.0008
0.003 2.98E-02
0.01 N/S
N/S 0.00002
1E-35 0.003
1E-21 0.0001
6E-10 N/S
0.005 N/S
0.00000001 N/S
9E-09 1.00E-07
1E-21 0.0008
N/S 3.00E-07
0.001 N/S
Normal
Tumour
OCCAMS
UALCAN
0
5
10
15
log2(TPM+1)


B
Vo−ATPase ATP6V0A1
9.20e−05 Vo−ATPase ATP6V0A2
6.13e−04 Vo−ATPase ATP6V0A4
4.67e−03 Vo−ATPase ATP6V0B
2.93e−06 Vo−ATPase ATP6V0C
Voltage−gated proton channel HVCN1
1.63e−09 Proton exchangers SLC9A1
3.15e−07 Proton exchangers SLC9A2
1.60e−09 Proton exchangers SLC9A3
3.76e−02 Proton exchangers SLC9A4
9.66e−13 Proton exchangers SLC9A5
Proton exchangers SLC9A6
5.36e−05 Proton exchangers SLC9A7
1.76e−03 Proton exchangers SLC9A8
3.27e−06 Proton exchangers SLC9A9
Sodium−bicarbonate exchangers SLC4A1
4.30e−07 Sodium−bicarbonate exchangers SLC4A2
1.08e−23 Sodium−bicarbonate exchangers SLC4A3
5.13e−04 Sodium−bicarbonate exchangers SLC4A4
1.59e−09 Sodium−bicarbonate exchangers SLC4A5
3.63e−02 Sodium−bicarbonate exchangers SLC4A7
5.25e−35 Sodium−bicarbonate exchangers SLC4A8
Sodium−bicarbonate exchangers SLC4A9
2.16e−05 Sodium−bicarbonate exchangers SLC4A10
Sodium−bicarbonate exchangers SLC4A11
5.25e−14 Chloride−bicarbonate exchangers SLC26A1
9.10e−03 Chloride−bicarbonate exchangers SLC26A2
5.24e−04 Chloride−bicarbonate exchangers SLC26A3
5.66e−04 Chloride−bicarbonate exchangers SLC26A4
2.98e−09 Chloride−bicarbonate exchangers SLC26A5
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3.60e−07 Chloride−bicarbonate exchangers SLC26A7
1.94e−15 Chloride−bicarbonate exchangers SLC26A8
8.18e−27 Chloride−bicarbonate exchangers SLC26A9
3.16e−04 Chloride−bicarbonate exchangers SLC26A10
6.47e−11 Carbonic anyhydrases CA1
7.66e−03 Carbonic anyhydrases CA2
4.13e−05 Carbonic anyhydrases CA3
4.89e−13 Carbonic anyhydrases CA4
Carbonic anyhydrases CA6
2.44e−10 Carbonic anyhydrases CA7
8.13e−28 Carbonic anyhydrases CA8
2.23e−36 Carbonic anyhydrases CA9
Carbonic anyhydrases CA10
4.16e−02 Carbonic anyhydrases CA11
2.61e−04 Carbonic anyhydrases CA12
Carbonic anyhydrases CA13
1.49e−04 Carbonic anyhydrases CA14
Upregulated
Downregulated
Normal tissue
OAC tissue
0
5
10
15
log2(TPM+1)

Figure 2.2. Expression of A) Acid-sensing channels, receptors and their accessory proteins; and of proton-regulating proteins (B) in OAC

Heat maps depicting the expression levels of genes encoding proteins involved in acid-sensing and proton homeostasis in OAC tumor *versus* normal tissue. The heat map shows expression data for patients from the OCCAMS dataset. The look-up table represents expression in TPM, with log2 (TPM+1) scaling. The *P*-values for significant expression levels (significance was set at $P < 0.05$) from each dataset are listed on the right, with *P*-values in red font indicating upregulation, and *P*-values in blue font indicating downregulation, compared to normal tissue. The *P*-values highlighted in yellow were considered significant after adjustment for MOT. N/S indicates a non-significant change in expression levels in tumor *versus* normal tissue.

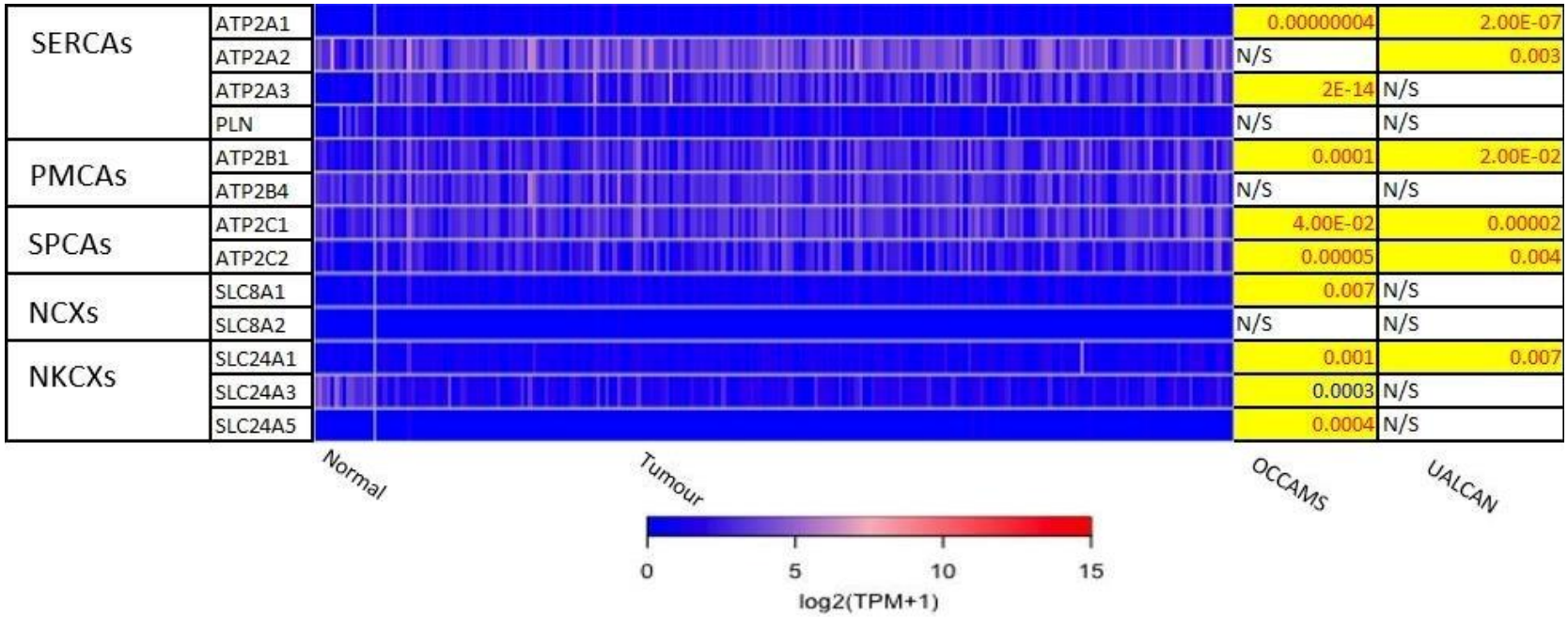
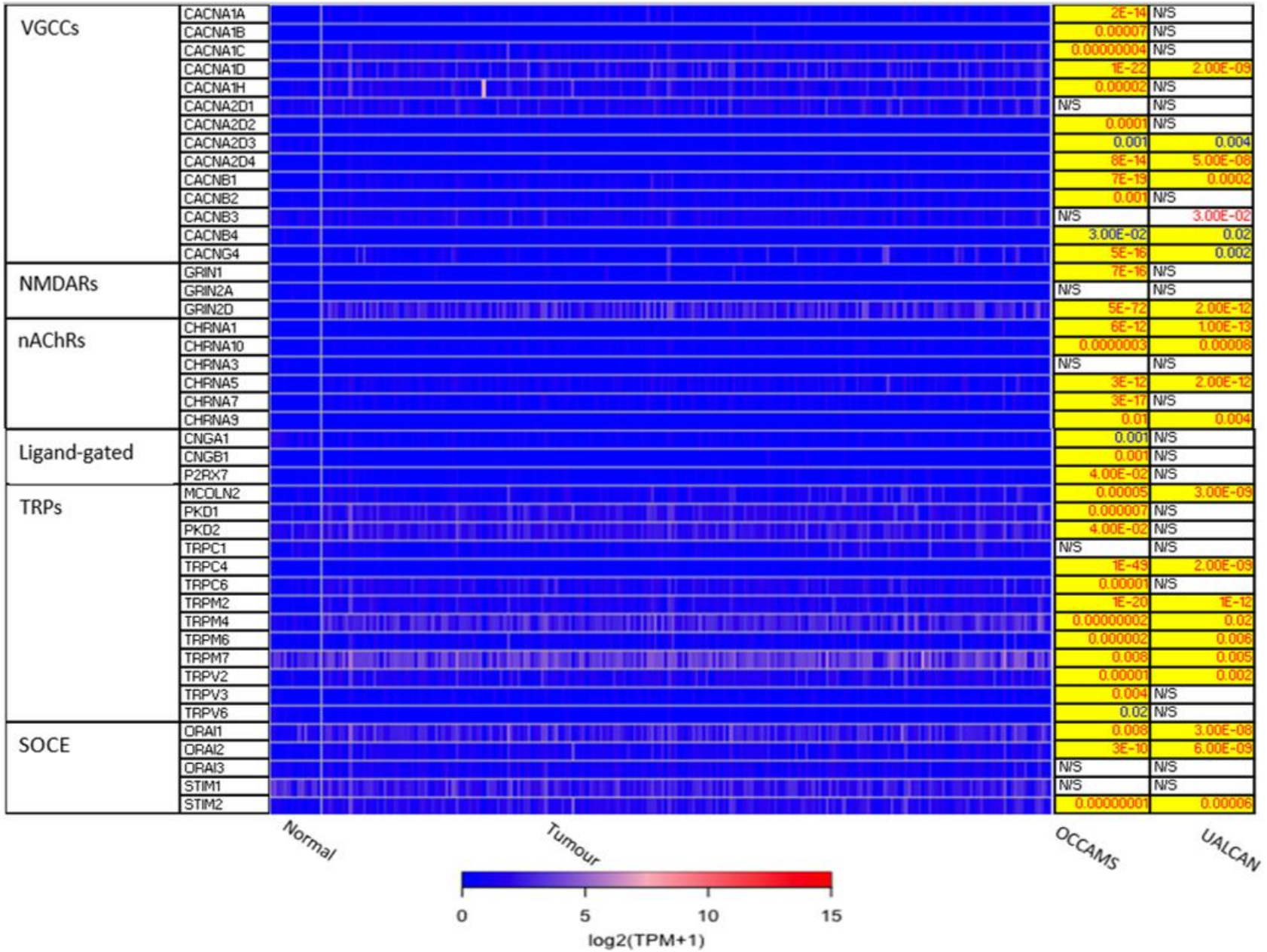


Figure 2.3. Expression of Ca^{2+} pumps and exchangers in OAC. Please see Figure 2.2 for details


VGCCs
CACNA1A
CACNA1B
CACNA1C
CACNA1D
CACNA1H
CACNA2D1
CACNA2D2
CACNA2D3
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CACNB1
CACNB2
CACNB3
CACNB4
CACNG4
NMDARs
GRIN1
GRIN2A
GRIN2D
nAChRs
CHRNA1
CHRNA10
CHRNA3
CHRNA5
CHRNA7
CHRNA9
Ligand-gated
CNGA1
CNGB1
P2RX7
TRPs
MCOLN2
PKD1
PKD2
TRPC1
TRPC4
TRPC6
TRPM2
TRPM4
TRPM6
TRPM7
TRPV2
TRPV3
TRPV6
SOCE
ORAI1
ORAI2
ORAI3
STIM1
STIM2
2E-14 N/S
0.00007 N/S
0.00000004 N/S
1E-22 2.00E-09
0.00002 N/S
N/S N/S
0.0001 N/S
0.001 0.004
8E-14 5.00E-08
7E-19 0.0002
0.001 N/S
N/S 3.00E-02
3.00E-02 0.02
5E-16 0.002
7E-16 N/S
N/S N/S
5E-72 2.00E-12
6E-12 1.00E-13
0.0000003 0.00008
N/S N/S
3E-12 2.00E-12
3E-17 N/S
0.01 0.004
0.001 N/S
0.001 N/S
4.00E-02 N/S
0.00005 3.00E-09
0.000007 N/S
4.00E-02 N/S
N/S N/S
1E-49 2.00E-09
0.00001 N/S
1E-20 1E-12
0.00000002 0.02
0.000002 0.006
0.008 0.005
0.00001 0.002
0.004 N/S
0.02 N/S
0.008 3.00E-08
3E-10 6.00E-09
N/S N/S
N/S N/S
0.00000001 0.00006
Normal
Tumour
OCCAMS
UALCAN
0
5
10
15
log2(TPM+1)

Figure 2.4. Expression of Ca^{2+} channels in OAC

MCOLN1 was not available from the OCCAMS data. *MCOLN1* was upregulated, compared to normal tissue, in the UALCAN data (*P* = 9.95E-4). Please see Figure 2.2 for detail

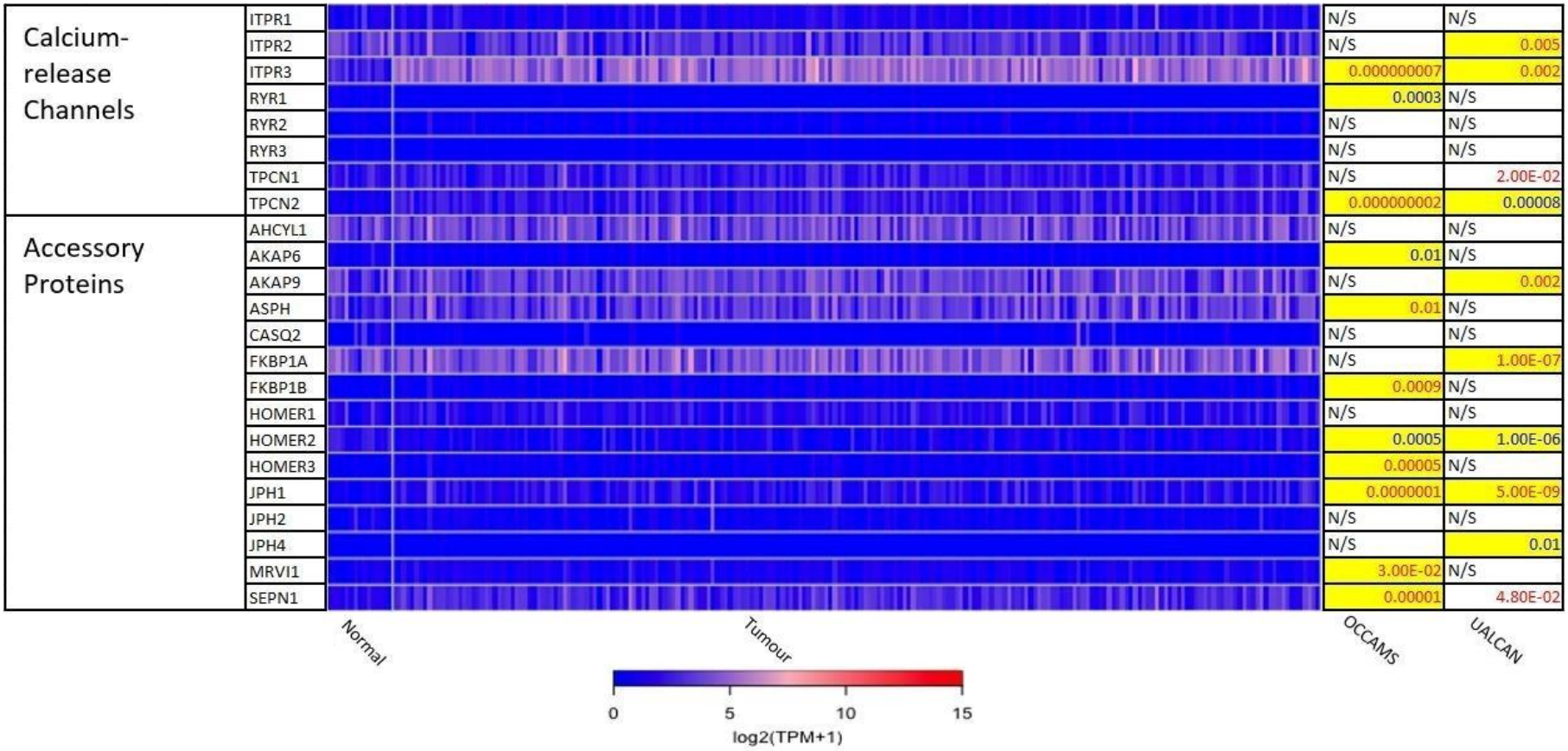

Figure 2.5. Expression of Ca^{2+}-release channels and their accessory proteins in OAC

JPH3 was not available from the OCCAMS data and was neither significantly upregulated nor downregulated in UALCAN. For details, please see Figure 2.2.

Further analysis of the 75 genes (significantly differentially expressed across both datasets) revealed that expression of *GRIN2D*, *TRPC4,* and *TRPM2* ranked the highest in terms of weighted significance. The individual *P*-values from the UALCAN data for these genes ranged from between to 2e-9 to 1e-12. The corresponding *P*-values from the OCCAMS dataset were: *GRIN2D*, 5e-72; *TRPC4*, 1e-49; and *TRPM2*, 1e-20.

Survival analysis and grade and stage expression analysis

Kaplan-Meier log-rank tests on the 271 genes from the OCCAMS dataset revealed that 21 had significant associations with survival (all were associated with improved survival with higher expression). These 21 genes were also analysed using Kaplan-Meier log-rank tests in the UALCAN portal, but none proved significant. Of the 21 genes significantly associated with survival, 9 had significantly, altered, mRNA-expression levels (as assessed by weighted ranking) in both datasets. The top 6 of these 9 genes were shortlisted for further analysis. Listed in order of their statistical significance (weighted rank of mRNA-expression levels in both datasets), these survival-associated genes were: *CACNA1D*, *CACNA2D4*, *JPH1*, *ACCN4*, *TRPM5,* and *ATP2C2*. Kaplan-Meier survival plots for these 6 genes are shown in Figures 2.6, 2.8, 2.9, 2.11, 2.12, and 2.13, respectively. *CACNA1D*, *JPH1,* and *ATP2C2* were also consistently upregulated in various OAC grades and metastatic stages across both datasets. ANOVA boxplots for *CACNA1D*, *JPH1,* and *ATP2C2* are shown in Figures 2.7, 2.10, and 2.14, respectively. *CACNA2D4* was consistently upregulated in various metastatic stages across both datasets, but not in OAC grades (Figure S6). *ACCN4* was consistently upregulated in various OAC-tumor grades across both datasets, but not in OAC metastatic stages (Figure S7). *TRPM5* only showed significant upregulation in OAC-tumor grades and metastatic stages in the OCCAMS dataset (Figure S8). Despite the importance of the mitochondrion in Ca^{2+} signalling, we did not find any

association of the 13 genes encoding mitochondrial proteins examined with survival outcomes.

CACNA1D

CACNA1D encodes the α_{1D} subunit of the L-type VGCC or $Ca_v1.3$. The channel mediates the influx of Ca^{2+} into the cell, upon membrane depolarization [85]. It is found in smooth, muscle cells, skeletal muscle, ventricular myocytes, bone (osteoblasts), the brain, the kidneys, the pancreas, the ovaries, the retina, and the cochlea [85, 86]. Patients with higher *CACNA1D*-expression lived longer than those with lower expression levels (log-rank *P* = 0.018), Figure 2.6. *CACNA1D*-expression was upregulated in Grade 3 *versus* Normal (*P* = 3.77e-2) in the UALCAN portal and in Grades 1, 2, and 3 *versus* Normal in the OCCAMS dataset (*P* = 2.00e-3, *P* = 8.40e-6, and *P* = 1.43e-5, respectively), Figure 2.7. *CACNA1D*-expression was upregulated in the N1, nodal-metastatic stage *versus* Normal (*P* = 3.30e-2) in the UALCAN portal and in Stages N0, N1, and N2 in the OCCAMS dataset (*P* = 6.90e-6, *P* = 4.58e-5 and *P* = 6.15e-4, respectively), Figure 2.7.

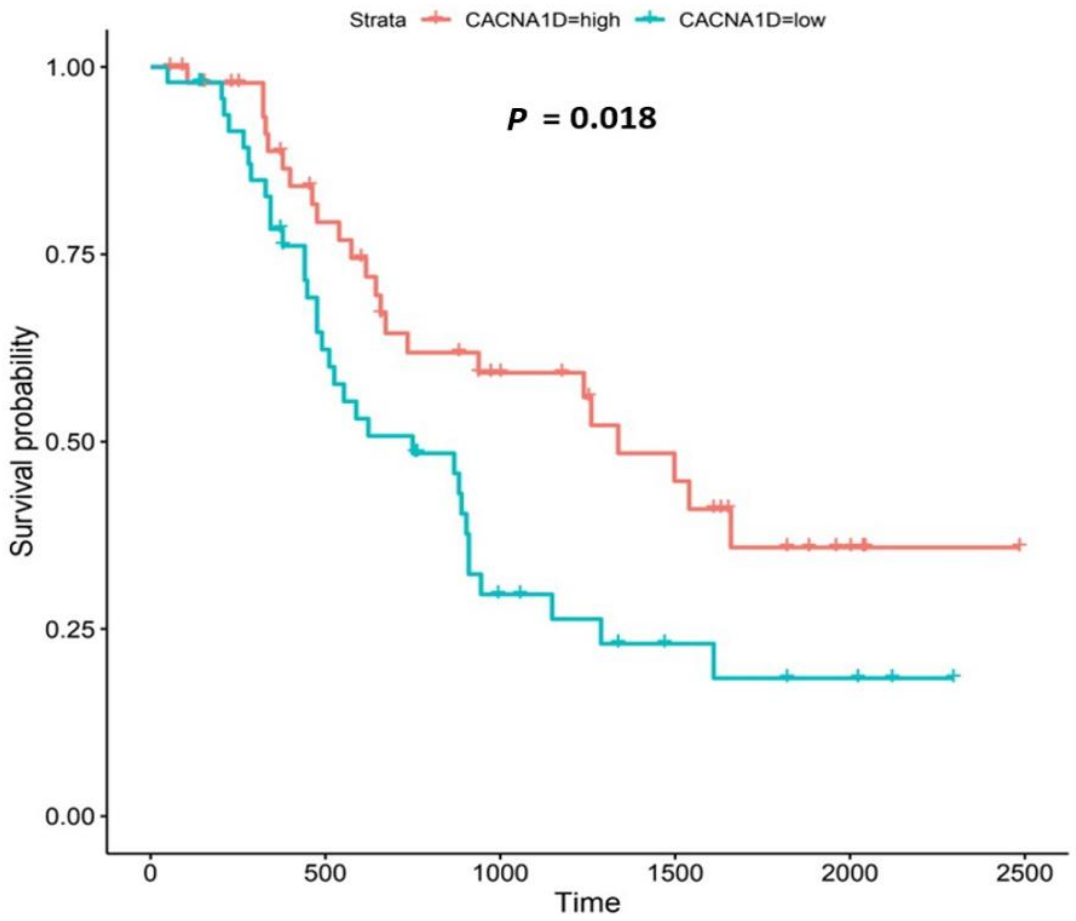

Figure 2.6. Kaplan-Meier survival plot for *CACNA1D*

Kaplan-Meier, survival plot (OCCAMS data) for *CACNA1D*. The comparison was made between patients with high *CACNA1D*-expression (TPM above the upper quartile) and those with low expression (TPM below the lower quartile). The plot shows survival probability with increasing time in days. A log-rank *P*-value of < 0.05 was considered statistically significant. An adjustment for MOT (FDR Method) was made.

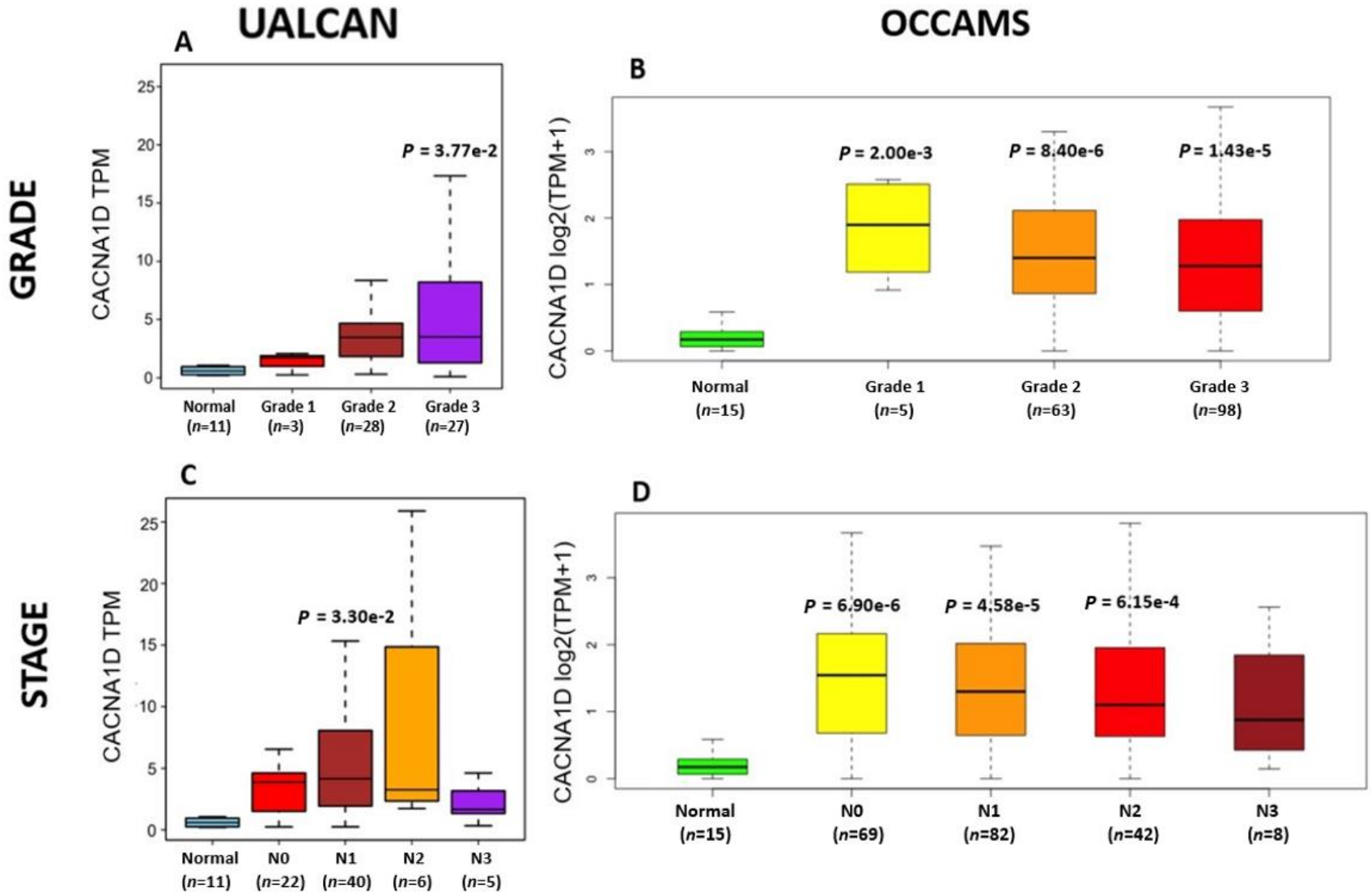
UALCAN
OCCAMS
GRADE
STAGE
A
B
C
D
CACNA1D TPM
CACNA1D log2(TPM+1)
P = 3.77e-2
P = 2.00e-3
P = 8.40e-6
P = 1.43e-5
P = 3.30e-2
P = 6.90e-6
P = 4.58e-5
P = 6.15e-4
Normal (n=11)
Grade 1 (n=3)
Grade 2 (n=28)
Grade 3 (n=27)
Normal (n=15)
Grade 1 (n=5)
Grade 2 (n=63)
Grade 3 (n=98)
Normal (n=11)
N0 (n=22)
N1 (n=40)
N2 (n=6)
N3 (n=5)
Normal (n=15)
N0 (n=69)
N1 (n=82)
N2 (n=42)
N3 (n=8)

Figure 2.7. OAC tumor grade and nodal metastatic stage boxplots for *CACNA1D*

A) (UALCAN data) and B) (OCCAMS data) show *CACNA1D*-expression across various OAC-tumor grades, compared to normal tissue. C) (UALCAN data) and D) (OCCAMS data) show *CACNA1D*-expression across various nodal-metastatic stages, compared to normal tissue. A *P*-value of < 0.05 was considered statistically significant for the boxplots. Tukey HSD *post hoc* tests were adjusted for MOT. The number of tumor tissue, normal tissue (OCCAMS), or NAT (UALCAN) samples is represented by *n*.

CACNA2D4

CACNA2D4 encodes the α2 and δ4 subunits of the L-type VGCC, Cav1.4. Like Cav1.3, the Cav1.4 channel mediates the influx of Ca^{2+} into the cell upon membrane depolarization [87]. Patients with higher *CACNA2D4*-expression lived longer than those with lower expression (log-rank *P* = 0.018), Figure 2.8.

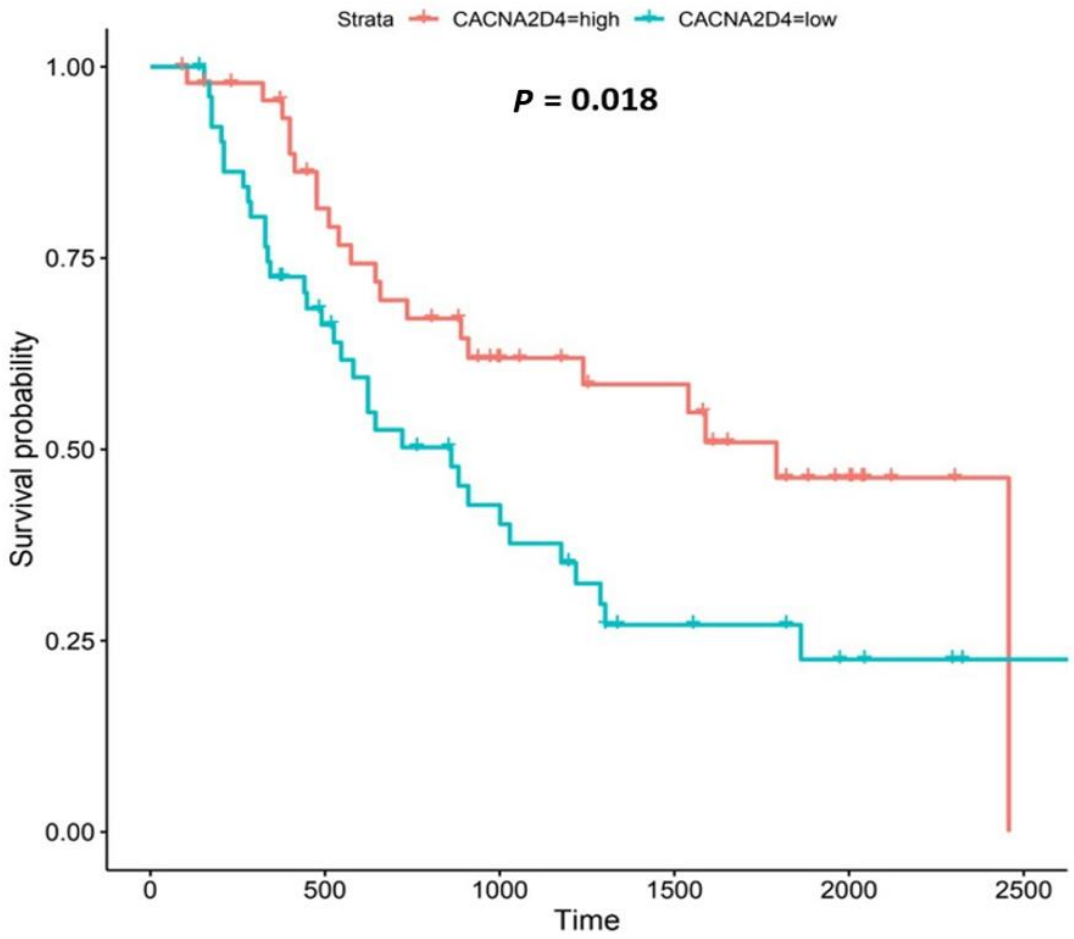

Figure 2.8. Kaplan-Meier survival plot for *CACNA2D4*

Details as described in Figure 2.6

JPH proteins

The JPH proteins contribute to $[Ca^{2+}]_c$ homeostasis by forming junctional membrane complexes [88]. Patients with higher *JPH1*-expression lived longer than those with lower expression (log-rank *P* = 0.019), Figure 2.9. *JPH1* was upregulated in various OAC-tumor grades in both datasets (Grade 3 *versus* Normal, *P* = 3.356e-2 for UALCAN and in Grades 1, 2 and 3 *versus* Normal, *P* = 8.98e-5, *P* = 2.24e-4 and *P* = 7.07e-5 for OCCAMS), Figure 2.10. *JPH1* was upregulated in various OAC nodal-metastatic stages in both datasets (N0 *versus* Normal, *P* = 4.35e-2 for UALCAN and N0, N1, N2 and N3 *versus* Normal, *P* = 1.70e-5, *P* = 1.80e-3, *P* = 2.44e-4 and *P* = 4.44e-2 for OCCAMS), Figure 2.10.

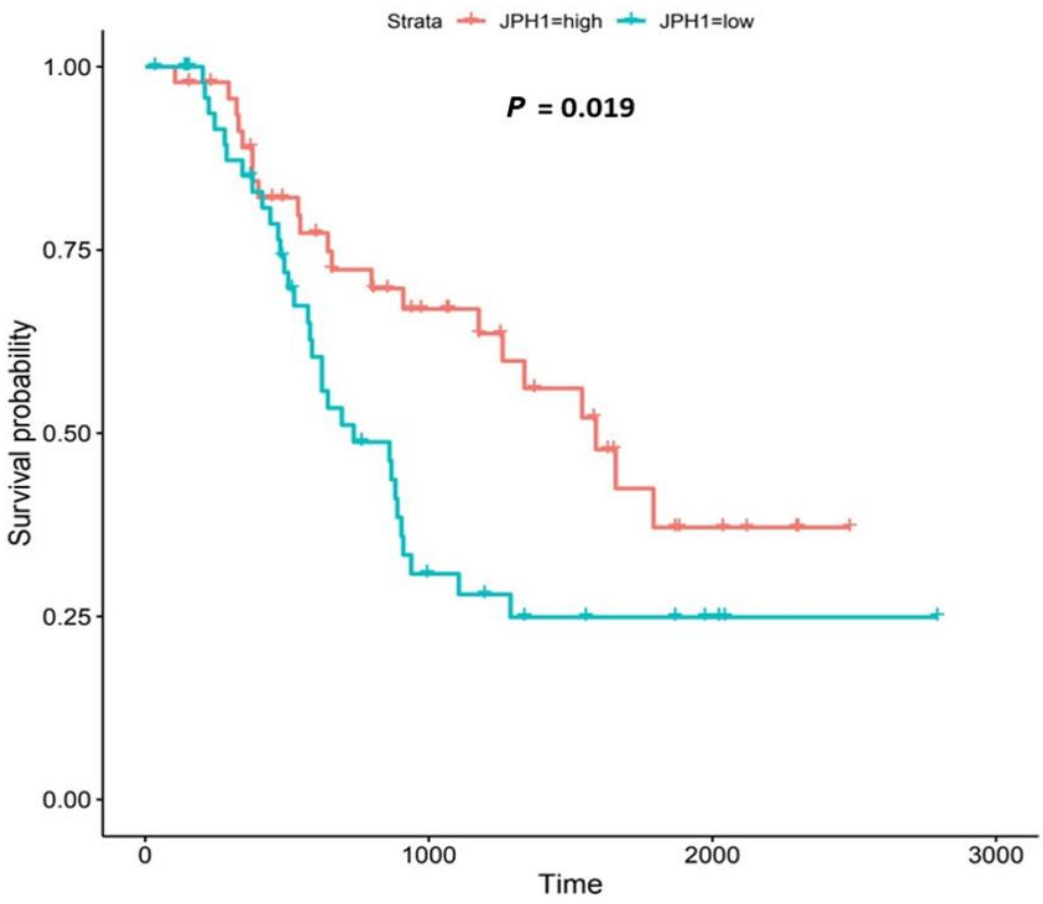

Figure 2.9. Kaplan-Meier survival plot for *JPH1*

Details as described in Figure 2.6

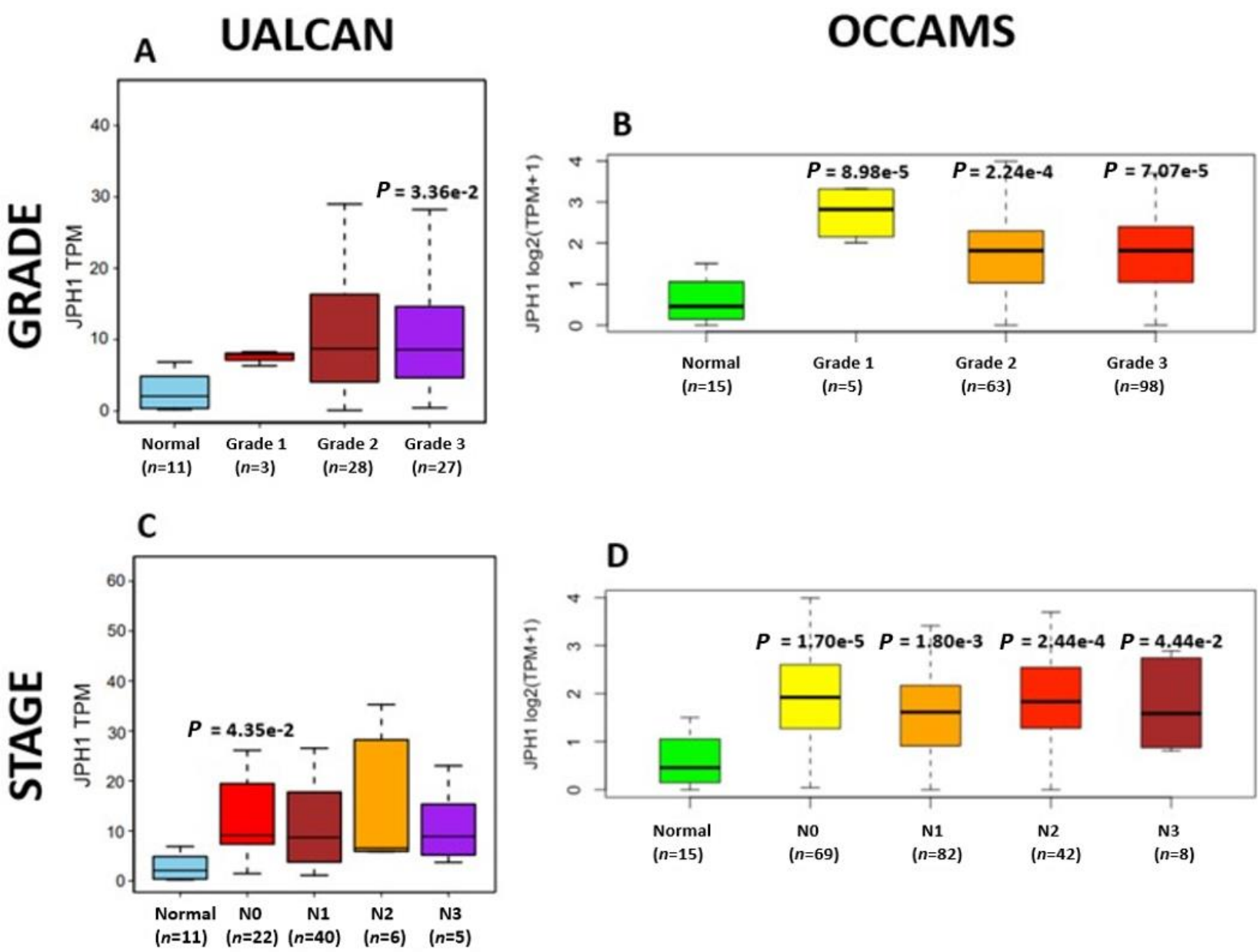
UALCAN
OCCAMS
A
B
C
D
GRADE
STAGE
JPH1 TPM
JPH1 log2(TPM+1)
P = 3.36e-2
P = 8.98e-5
P = 2.24e-4
P = 7.07e-5
P = 4.35e-2
P = 1.70e-5
P = 1.80e-3
P = 2.44e-4
P = 4.44e-2
Normal (n=11)
Grade 1 (n=3)
Grade 2 (n=28)
Grade 3 (n=27)
Normal (n=15)
Grade 1 (n=5)
Grade 2 (n=63)
Grade 3 (n=98)
Normal (n=11)
N0 (n=22)
N1 (n=40)
N2 (n=6)
N3 (n=5)
Normal (n=15)
N0 (n=69)
N1 (n=82)
N2 (n=42)
N3 (n=8)

Figure 2.10. OAC tumor grade and nodal metastatic stage boxplots for *JPH1*

Details as described in Figure 2.7

ACCN4

ACCN4 encodes the proton-gated, amiloride-sensitive, cation channel, ASIC4, which has been linked to synaptic transmission, pain perception, and mechano-perception [89, 90]. Patients with higher *ACCN4*-expression lived longer than those with lower expression (log-rank *P* = 0.0085), Figure 2.11.

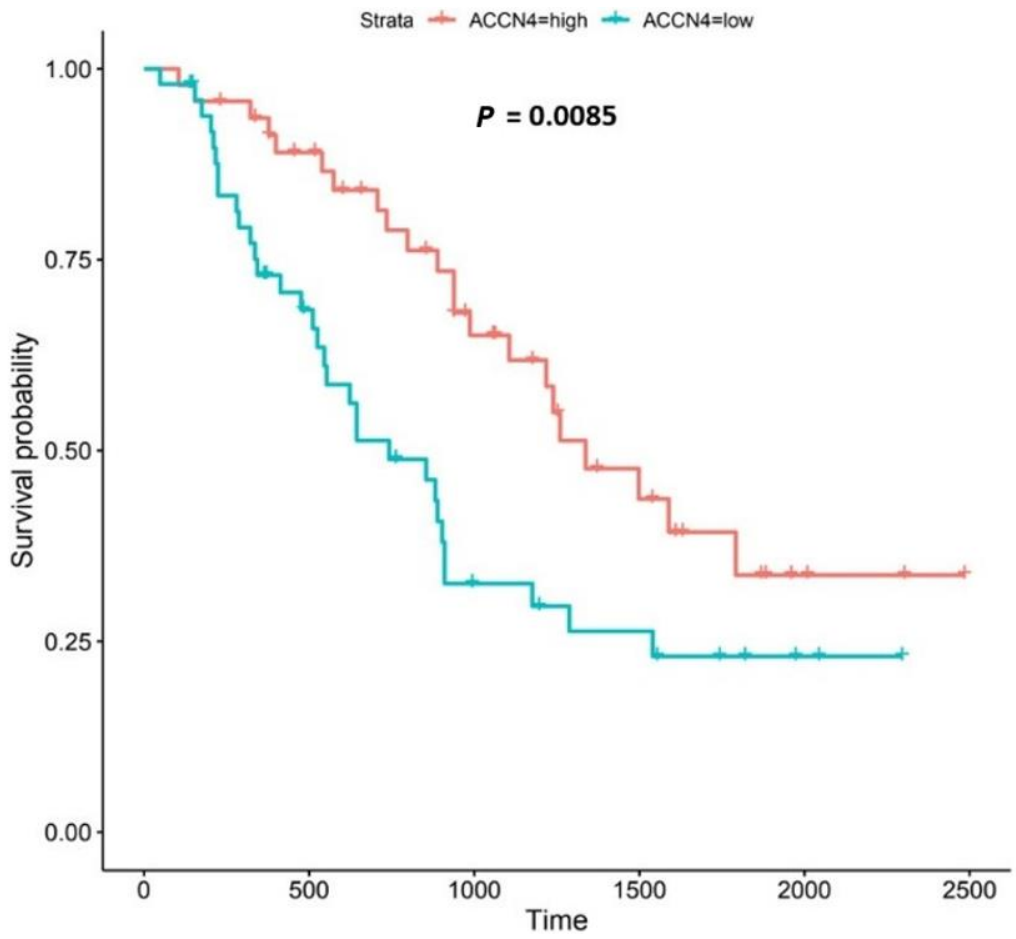

Figure 2.11. Kaplan-Meier survival plot for *ACCN4*

Details as described in Figure 2.6

TRPM5

TRPM5 is a member of the TRP superfamily of ion channels [91]. Patients with higher *TRPM5* expression lived longer than those with lower expression (log-rank *P* = 0.026), Figure 2 . 12.

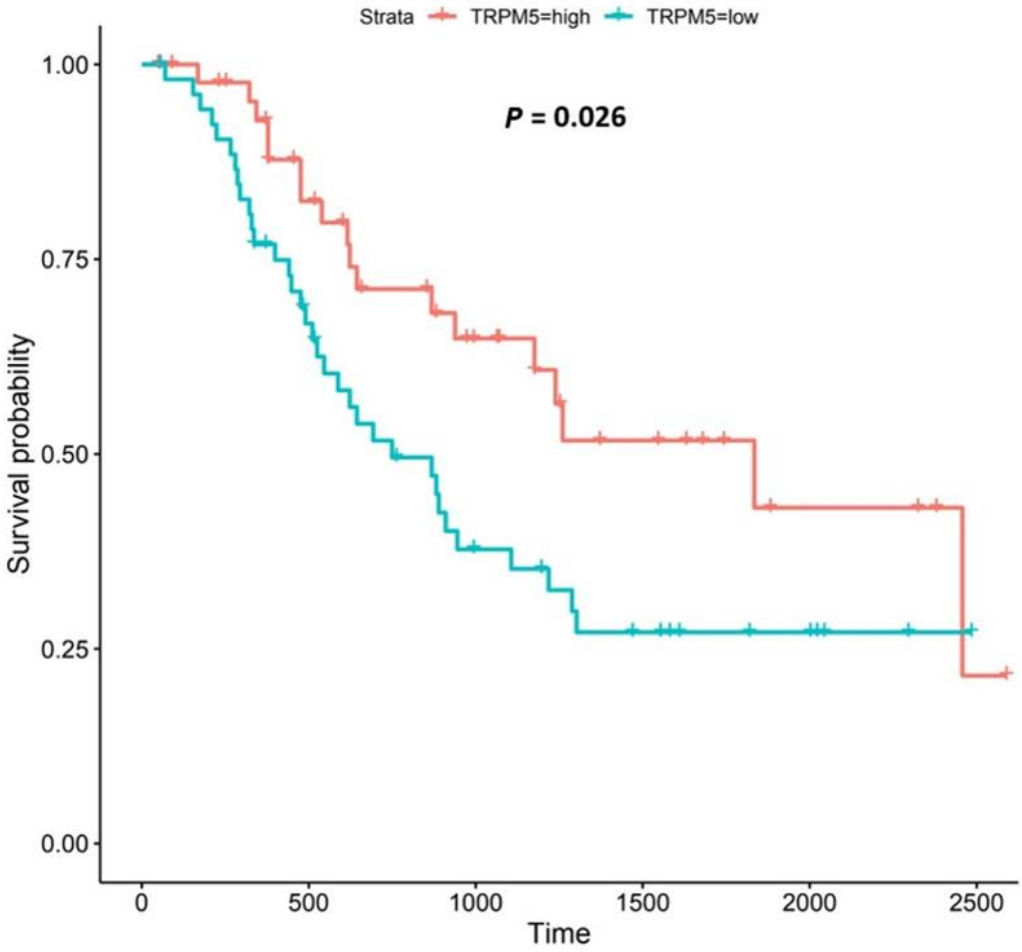

Figure 2.12. Kaplan-Meier survival plot for *TRPM5*

Details as described in Figure 2.6.

ATP2C2

ATP2C2 encodes an ATPase which transports Ca^{2+} and Mn^{2+} into the Golgi lumen to regulate protein sorting, processing, and glycosylation [92]. Patients with higher *ATP2C2*-expression lived longer than those with lower expression (log-rank *P* = 0.0018), Figure 2.13. *ATP2C2* was upregulated in various OAC-tumor grades in both datasets examined (Grade 2 *versus* Normal, *P* = 2e-2 for UALCAN and in Grades 1, 2 and 3 *versus* Normal, *P* = 9.6e-3, *P* =3.8e-5 and *P* =4.3e-4 for OCCAMS), Figure 2.14.

ATP2C2 was also upregulated in various nodal-metastatic stages in both datasets (N0 *versus* Normal, *P* =5e-2 for UALCAN and in N0, N1 and N2 *versus* Normal, *P* =3e-4, *P* =1e-3 and *P* = 8e-3 for OCCAMS), Figure 2.14.

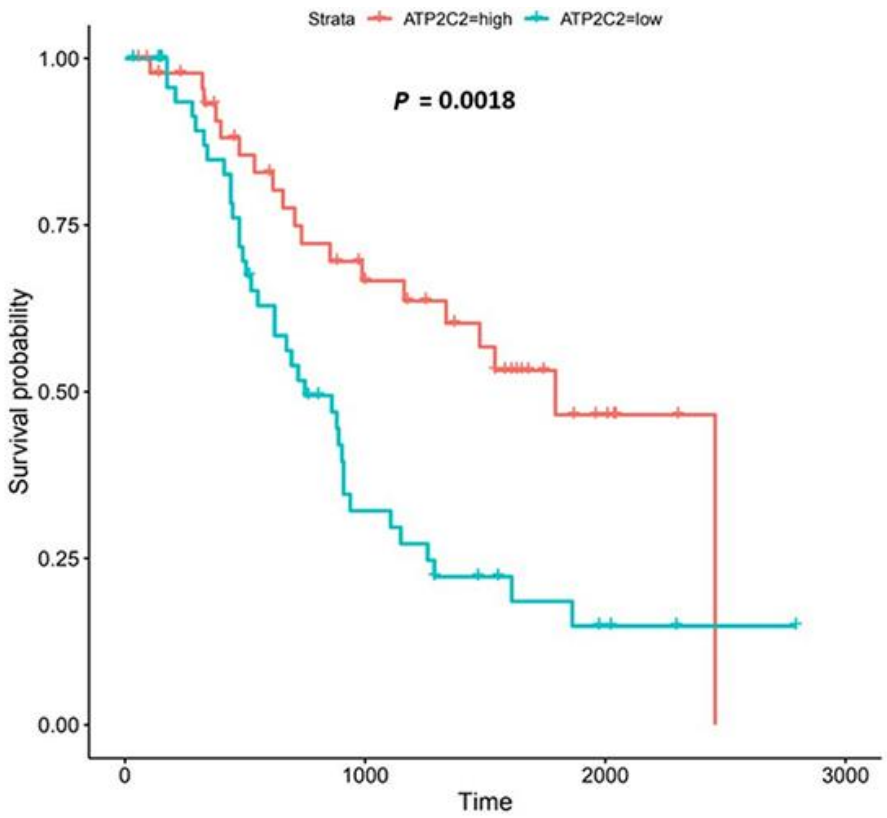

Figure 2.13 Kaplan-Meier survival plot for *ATP2C2*

Details as described in Figure 2.6

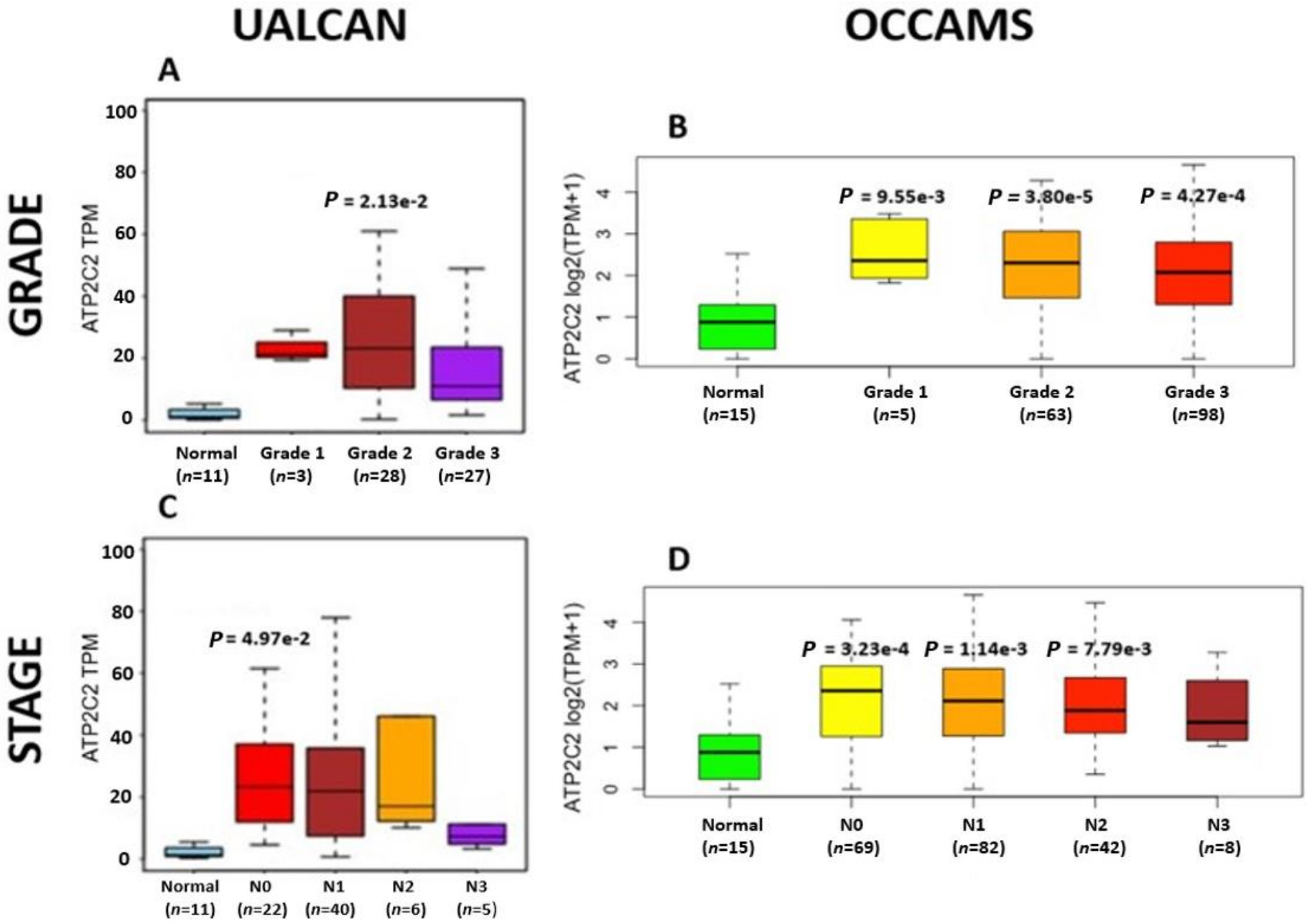
UALCAN
OCCAMS
GRADE
STAGE
A
B
C
D
ATP2C2 TPM
ATP2C2 log2(TPM+1)
P = 2.13e-2
P = 9.55e-3
P = 3.80e-5
P = 4.27e-4
P = 4.97e-2
P = 3.23e-4
P = 1.14e-3
P = 7.79e-3
Normal (n=11)
Grade 1 (n=3)
Grade 2 (n=28)
Grade 3 (n=27)
Normal (n=15)
Grade 1 (n=5)
Grade 2 (n=63)
Grade 3 (n=98)
Normal (n=11)
N0 (n=22)
N1 (n=40)
N2 (n=6)
N3 (n=5)
Normal (n=15)
N0 (n=69)
N1 (n=82)
N2 (n=42)
N3 (n=8)

Figure 2.14. OAC tumor grade and nodal metastatic stage boxplots for *ATP2C2*

Details as described in Figure 2.7

5. Discussion

The goal of the present study was to examine whether there are alterations in the Ca^{2+} toolkit during the progression of OAC and if altered gene expression is associated with patient outcome. We particularly focused on Ca^{2+}-toolkit proteins involved in acid-sensing and on Ca^{2+}-toolkit proteins located in the mitochondrion. Ca^{2+} is a key second messenger which regulates most aspects of cell biology, including gene transcription, cellular secretion, cellular motility, and cell death [12]. In cancer cells, dysregulated Ca^{2+}-signalling can lead to transformation, proliferation, and migration [21]. Increases in $[Ca^{2+}]c$ can be elicited by decreases in extracellular pH [10]. During acid reflux, the pH of the distal oesophagus can drop from about pH 6.5 to approximately pH 2 [93]. EA acts as a signal for the transformation of squamous to glandular cells in BO, a precursor to OAC [94]. The potential link between Ca^{2+} signalling and oncogenesis in OAC, and if such oncogenesis can be stimulated by EA, is largely unexplored and offers opportunities for the development of new chemotherapeutic approaches. Such strategies would target EA-related pathways in both pre-malignant disease and OAC.

The present transcriptomic analysis employed two different patient datasets. Our key findings are that *CACNA1D*, *CACNA2D4*, *JPH1*, *ACCN4*, *TRPM5*, and *ATP2C2* were significantly associated with improved survival and were also significantly upregulated compared to normal tissue in both datasets. Furthermore, there were significant differences in the transcription levels of *CACNA1D*, *JPH1*, and *ATP2C2* between various tumor grades and nodal-metastatic stages in both datasets. Finally, *GRIN2D*, *TRPC4*, and *TRPM2* were the most differentially expressed genes, based on weighted rank for significance-level, in both datasets.

CACNA1D encodes the α_{1D} subunit of the L-type VGCC, $Ca_v1.3$. Currently, there is no evidence in the literature linking *CACNA1D* to acid-sensing. *CACNA1D*-expression was upregulated compared to normal tissue in both datasets investigated in the present

study. Furthermore, high *CACNA1D*-expression was associated with improved survival outcomes. *CACNA1D*-expression was upregulated in Grade 3 *versus* normal tissue in the UALCAN portal and in Grades 1, 2, and 3 *versus* normal tissue in the OCCAMS dataset. *CACNA1D*-expression was upregulated in the second (N1) nodal-metastatic stage compared to normal tissue in the UALCAN portal and in the N0, N1, and N2 stages in the OCCAMS dataset. Other *CACNA1D*-expression-related studies have shown conflicting results on the effect of levels of this gene in cancers. Most studies have been carried out on prostate cancers, which contain the transmembrane protease, serine 2, gene-erythroblast transformation-specific-related gene (*TMPRSS2-ERG*) gene fusion. A bioinformatics meta-analysis of the Oncomine dataset (Wang et al. [95]) revealed that various subtypes of VGCCs (*CACNA1D* or $Ca_v1.3$ included) were implicated in the development and progression of diverse types of cancer, including cancer of the prostate, breast, colorectum, bladder, stomach, lung, brain, uterus and oesophagus [95]. The same study reported low *CACNA1D*-expression in sarcoma and renal tumours. Biasiotta et al. [96] noted that *CACNA1D* showed significantly increased expression in at least 13 of the 25 bladder-cancer datasets analysed; these bladder-cancer results reinforce those from the meta-analysis by Wang et al. A Ca^{2+}-toolkit-specific transcriptomic analysis (Pérez-Riesgo et al. [97]) revealed that *CACNA1D*-expression was increased 1.55-fold in human colorectal cancer cells, compared to normal colon cells. In endometrial carcinoma, *CACNA1D* was also upregulated [98]. In a study of radical prostatectomy patients, *CACNA1D*-expression was correlated with a higher Gleason score (a grading system for prostate cancer) and biochemical recurrence [99]. An analysis of the Oncomine dataset revealed that *CACNA1D*-expression was significantly higher in prostate cancers with the *ERG*-gene fusion, compared with the cases without this gene fusion [100]. Jhavar et al. [101] observed that *CACNA1D* was among the top ten differentially-expressed genes in the ERG-subtype of prostate cancer, compared to samples lacking ERG-expression. Setlur et al. [102] identified *CACNA1D* as part of an 87 gene signature for ERG-fusion-bearing prostate cancer. An epigenomic-profiling study of prostate cancer tumours noted that *CACNA1D* was among the top-ten-ranked differentially-methylated (hypomethylated) genes in tissues with ERG fusion, compared to those without [103]. *CACNA1D*, mRNA-expression was also inversely

correlated with methylation of the gene [103]. Phan et al. [104] used Oncomine to calculate the changes in mRNA-expression of VGCCs in 20 types of cancer: in contrast to our findings, *CACNA1D* exhibited low expression in the brain, kidney, and lung tumours [104].

Analyses relating to the $Ca_v1.3$ protein are consistent with our gene-expression findings. Fourbon et al. [105] demonstrated that the $Ca_v1.3$ protein was more abundant in colorectal-cancer biopsies, compared to normal tissue. Chen et al. [100] noted that the $Ca_v1.3$ protein was more abundant in prostate cancer and modulated androgen receptor transactivation. Furthermore, the use of Ca^{2+}-channel blockers (dihydropyridines, phenylalkylamines, and benzothiazepines) was associated with a reduced risk for a higher Gleason score and ERG-positive prostate cancer [106]. Two studies focused on the association of *CACNA1D* with survival outcomes [107, 108]. Wang et al. [107] identified *CACNA1D* as part of an 18-ion-channel, prognostic-gene signature in glioma (the direction of the association with survival outcomes was not reported); this finding was observed even though *CACNA1D*-expression was downregulated in these cells. Xing et al. [108] noted that *CACNA1D* was one of 8 out of 3,747 differentially-expressed genes associated with survival outcomes in colon adenocarcinoma: consistent with our findings, those with higher *CACNA1D*-expression lived longer than those with lower expression [108]. Similar to our metastasis-related findings, *CACNA1D* was among the 6 genes associated with tumor node metastasis staging in colon adenocarcinoma [108]. Additionally, Fourbon et al. [105] showed that colon cancer cell migration was affected by *CACNA1D*-expression: the migration was decreased when *CACNA1D* was silenced.

The observation that *CACNA1D* was upregulated in OAC, but was also associated with improved patient survival, is of interest. Such associations with improved survival may be due to the potential effect of *CACNA1D* on Ca^{2+}-dependent cancer-cell death. VGCC-associated (particular Ca_v1-associated), Ca^{2+}-dependent cell death has been well described in pancreatic β cells [109], but to date, not in OAC. Several cancer-predisposing mechanisms have been linked to VGCC function, including the direct influx of Ca^{2+} into the cell, the involvement of VGCC subunits, and the involvement of

the steroid 17β-oestradiol [98, 105, 110].Ca^{2+} can enter the cytoplasm from the ER through the interaction between VGCCs and the RYR1, a process which facilitated by JPHs [111, 112]. The role of VGCC accessory subunits has been described in the mechanistic paragraph for *CACNA2D4*, below. The potential role of 17β-oestradiol in VGCC-associated carcinogenesis has been studied in endometrial carcinoma. Specifically, upregulation of *CACNA1D* was associated with increased proliferation and migration in endometrial carcinoma tissue. These effects were enhanced by 17β-oestradiol, via the G protein-coupled oestrogen receptor [98]. A previous study showed that these oestrogen-stimulated effects were decreased by Ca^{2+}-channel blockers (nifedipine and mibefradil) [110]. It is possible that any of the cancer-predisposing mechanisms of VGCCs mentioned could be inactivated in OAC, particularly if such inactivation was acid-dependent. Such inactivation would be consistent with the improved patient survival observed in the present study.

The *CACNA2D4* gene encodes the α2 and δ4 subunits of VGCC complexes and has been reported to be an oncogene [113]. *CACNA2D4* expression was upregulated compared to normal tissue in both OAC datasets. High *CACNA2D4*-expression was also associated with improved survival outcomes. *CACNA2D4* was upregulated compared to normal tissue in the fourth (N3), nodal-metastatic stage of OAC in the UALCAN portal, and in the first (N0) and third (N2) nodal-metastatic stages in the OCCAMS dataset. There is a paucity of literature investigating the role of *CACNA2D4* in OAC and in acid-sensing. A DNA-methylation study showed that *CACNA2D4* mRNA expression was upregulated in cultured gastric cancer cell lines, compared to normal stomach cells [87]. The role of *CACNA2D4* in pancreatic adenocarcinoma was examined by Xu et al. [114], using data from TCGA: in contrast to our findings, the authors noted a poor prognosis in a subset of patients with high *CACNA2D4*-expression. An analysis of 98 Ca^{2+}-regulating genes from two gene-expression-profiling datasets on gastric cancer, highlighted that *CACNA2D4* was associated with either a 40% decrease (Dataset One) or a 2.9-fold increase (Dataset Two) in overall survival [41]. Similar to our findings, *CACNA2D4* was one of the few genes associated with the metastasis of uveal melanoma [115].

Interestingly, the related *CACNA2D3* gene (encoding α2 and δ3 subunits) was downregulated in OAC tissue compared to normal tissue, in both the UALCAN and OCCAMS datasets; we did not, however, identify an association between *CACNA2D3*-expression and survival. Like *CACNA2D4*, research into the role of *CACNA2D3* in OAC and in acid-sensing is lacking. However, there have been a number of studies on OSCC: Li et al. [116] reported tumor-suppressor activity of *CACNA2D3* in OSCC cell lines and demonstrated that decreased expression in OSCC patients was associated with poor survival and enhanced metastasis. Increased *CACNA2D3*-expression has also been linked with enhanced chemosensitivity of OSCC to cisplatin [117]. An association between *CACNA2D3* and both patient survival and tumor metastasis has been demonstrated in other cancers [87, 118]. High *CACNA2D3*-expression was associated with improved survival outcomes in advanced gastric cancer: patients with detectable *CACNA2D3* gene methylation had a significantly shorter survival time than patients without this methylation [87]. Methylation-dependent transcriptional silencing of *CACNA2D3* was shown to contribute to the metastatic phenotype of oestrogen-receptor-positive primary breast cancer, again illustrating a protective nature of the gene [118]. We did not perform a metastasis-related analysis for *CACNA2D3* in our study because it did not show any significant association with survival. However, it could be useful to investigate whether *CACNA2D3* is a tumor suppressor in OAC. It would also be valuable to know whether there are any synergistic effects between *CACNA2D4* and *CACNA2D3*; the literature points to each gene having opposing roles in the cancers.

The observation that *CACNA2D4* upregulation was associated with improved patient survival warrants further study. The increased expression of VGCC accessory subunits, $\alpha_2\delta$ and β, has been related to different cancer hallmarks in liver, ovarian, prostate, pancreatic, lung, and colon tumours [119]. Supporting our observations, Wang et al. [113] showed that *CACNA2D4* played a role in mitigating the adverse effects of first-line chemotherapy (adriamycin or cisplatin) in the treatment of gastric cancers overexpressing bromodomain-containing protein 9 (BRD9). A mechanistic study has been carried out on *CACNA2D3*. Li et al. [116] demonstrated that *CACNA2D3* inhibited tumorigenicity by arresting the cell cycle at the G1/S checkpoint,

through increased p21 and p53 expression. Li et al. [116] also demonstrated that *CACNA2D3* inhibited cell motility and induced Ca^{2+}-dependent apoptosis. Similar to the *CACNA2D4* observations of Wang et al. [113], Nie et al. [117] noted that increased expression of *CACNA2D3* enhanced the chemosensitivity of OSCC to cisplatin via Ca^{2+}-mediated apoptosis and the suppression of the phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) pathway. The adverse effect of *CACNA2D3*-methylation (*CACNA2D3*-downregulation) has been described [87, 118]. Elucidating the effects of *CACNA2D4* on tumorigenicity, cell motility, apoptosis and chemosensitivity would be an invaluable addition to Ca^{2+} and OAC literature and would consolidate *CACNA2D3*-related literature.

JPH1 encodes the JPH1 protein [88]. The JPH proteins contribute to $[Ca^{2+}]_c$ homeostasis by forming junctional membrane complexes; this is achieved by anchoring the Sarco-/Endo-plasmic reticulum to the PM [88]. *JPH1* and *JPH2* are abundant in skeletal muscle and their suppression leads to the disruption of the activity of SOCE [88]. In our study, high *JPH1*-expression was associated with improved patient survival. Additionally, *JPH1*-expression was upregulated compared to normal tissue across both the UALCAN and OCCAMS datasets. Further analysis revealed that *JPH1* was upregulated across advanced OAC-tumor grades and OAC nodal-metastatic stages. There is no literature linking a role for *JPH1* in OAC or acid-sensing. *JPH1* was among the upregulated genes in an analysis of lung cancer [120]. Zou et al. [121] noted that the long non-coding form of *JPH1* RNA, Lnc-JPH1-7, was upregulated 35-fold in TCGA samples of head-and-neck, squamous-cell carcinoma. Low expression of this long non-coding RNA promoted survival, consistent with it suppressing the expression of the coding form of *JPH1* [121]. A study by Tsantoulis et al. [122] on uveal melanoma demonstrated that *JPH1*-expression was associated with relapse. By contrast, in uveal-melanoma tissue, *JPH1*-expression was downregulated compared to normal tissue [123]. An analysis by Que et al. [124] revealed that *JPH1* was one of 14 mRNA transcripts involved in regulating a microRNA-circRNA network of genes likely involved in the development or prevention of colorectal cancer [124]; whether *JPH1*-expression was upregulated or downregulated was not reported in this analysis. A mutation in the *JPH1* gene was noted in a patient with human T-

lymphotropic virus type-1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis who subsequently developed adult T-cell leukaemia [125]. *JPH1* was among the top 20 genes associated with survival in endometrial carcinoma [126]; whether *JPH1* was associated positively or negatively with survival was not reported. Consistent with our findings, *JPH1* was one of 14 of 7,222 genes identified as being strongly associated with a better prognosis in squamous-cell lung carcinoma [127]. Again, Zou et al. [121] noted associations between elevated Lnc-JPH1-7-levels and head-and-neck squamous-cell carcinoma: this time a link with poor prognosis was observed. The authors also highlighted a significant correlation between Lnc-JPH1-7 and the advanced, tumor stage [121]. Metastasis-related literature also aligns with our findings. Tsantoulis et al. [122] demonstrated that the expression of a combination of *JPH1* and the protein-tyrosine phosphatase 4A3 (*PTP4A3*) gene correlated with an increased risk of developing liver metastasis in colorectal and breast cancer (hormone-positive tumours only) [122]. Zou et al. [121] illustrated how short hairpin RNA-mediated knockdown of Lnc-JPH1-7 reduced the expression of epithelial-mesenchymal-transition-promoting genes in head-and-neck squamous-cell carcinoma cell lines.

Similar to *CACNA1D* and *CACNA2D4*, high *JPH1*-expression was associated with improved patient survival in our OAC study. A specific microRNA, miR-145, is the most compelling proposed underlying mechanism for this association with survival. miR-145 has been shown to regulate tumorigenesis, proliferation, differentiation, apoptosis, metastasis, angiogenesis, and therapeutic resistance in certain cancers [128, 129]. It has also been downregulated compared to normal tissue in several cancers, including OSCC. If upregulated, as in the case of OAC, miR-145 is typically accepted as a tumor-suppressor and a suppressor of therapeutic resistance [129]. Xu et al. [128] noted that *JPH1* was one of 78, potential targets of miR-145. If in the present study, *JPH1* is upregulated in OAC and associated with improved survival, it would suggest that miR-145 is interacting with *JPH1* post-transcriptionally to favour tumor suppression**.** Although calmodulin-dependent protein kinase 1D (*CAMK1D*) and calmodulin-dependent protein kinase 2D (*CAMK2D*) were also targeted by miR-145 in the study by Xu et al. [128], these proteins exhibited little significance in the

present study. Other studies have focused on *JPH2* and *JPH3*. *JPH2* was among 10 individual genes of a DNA-methylation signature associated with overall survival of gastric cancer patients [130]: in contrast to the *JPH1* observations of the present study, higher *JPH2*-methylation (gene-downregulation) was associated with longer survival [130]. In lung adenocarcinoma, *JPH3* was downregulated 0.2-fold by *S100A2* (associated with favorable prognosis in p53-negative tumours) and 0.43-fold by *S100A4* (associated with poor prognosis in p53-positive tumours) [131]. The potential role of the upregulation of *S100A2* and *S100A4* in the downregulation of *JPH1* was not consistently observed in the present study. Again, laboratory experimentation is required to verify the roles of *JPH1* in OAC.

ACCN4 encodes ASIC4 [89]. ASIC4 is thought to regulate other members of the ASIC family, particularly in the generation of pain-related currents [89]. These channels sense both transient and sustained acidification [90, 132]. In our study, we noted that *ACCN4* was upregulated in both the UALCAN and the OCCAMS datasets. High *ACCN4*-expression was associated with improved survival outcomes. Further analysis revealed that *ACCN4* was upregulated compared to normal tissue in advanced OAC-tumor grades (both datasets), and in the first (N0) nodal-metastatic stage in the OCCAMS dataset. There is no literature linking *ACCN4*-expression with OAC. Other cancer-related literature focuses on *ACCN4*-expression and metastasis, the results of which are conflicting. A study by Marques et al. [133] found that *ACCN4* was upregulated by r1881 (a synthetic androgen) in hormone-therapy-resistant prostate cancer cell lines. By contrast, *ACCN4* was overexpressed in cisplatin-sensitive ovarian cancer cells [134], suggesting a protective role of the gene. *ACCN4*-downregulation in head-and-neck squamous-cell carcinoma was noted by Braakhuis et al. [135]. A polysaccharide from the marine algae, *Gracilariopsis lemaneiformis,* (known for its anticancer activity) significantly decreased *ACCN4*transcription in a lung-cancer cell line [136]. In an analysis of the Oncomine dataset, focusing on 5 histologically distinct solid tumours (bladder cancer, glioblastoma, melanoma, breast, invasive-ductal cancer, and lung carcinoma), *ACCN4*-expression was neither upregulated nor downregulated, compared to normal tissue [96]. There is a paucity of literature associating *ACCN4*-expression with survival outcomes. Two studies highlighted a role

for *ACCN4* in metastatic tissue (one indicating a positive association and one indicating a negative association) [135, 137]. Di Pompo et al. [137] investigated whether breast cancer metastasis-induced, bone pain was associated with the effect of EA acting on the mesenchymal, tumor-associated stroma. The authors used human osteoblast primary cultures from healthy donors and cancer-associated fibroblasts from the tumor biopsies of patients with metastasis [137]. After exposure of both types of cells to a medium at pH 6.8 for 6 hours, they noted increased mRNA expression of *ACCN4* and *GPR65* [137]. They concluded that bone metastasis-associated mesenchymal cells have mechanisms in place to perceive the acidification of the metastasis microenvironment, leading to pain and that such findings may have implications for breast cancer palliative care [137]. In contrast to the study by Di Pompo et al. [137], Braakhuis et al.[135] noted that *ACCN4*-expression was downregulated in metastasized head-and-neck squamous-cell carcinoma compared to non-metastasized tissue.

ACCN4-upregulation led to improved survival outcomes in OAC in the present study. This is particularly interesting as the gene family has an established link to acid-sensing [90, 132]. Relative to other ASICs, however, literature on *ACCN4*-function is scarce. Zhou et al. [138] examined the molecular effects of *ACCN1, 2, 3, and 4* and noted that only ASIC2 (encoded by *ACCN1*) promoted invasion and metastasis of colorectal cancer, under acidosis; such metastasis was achieved by the activation of the calcineurin/NFAT1 axis [138]. The subtype of the calcineurin gene protein phosphatase 3 catalytic subunit alpha isoform (*PPP3CA*) and the *NFATC1* gene was upregulated only in the OCCAMS dataset in the present study. Zhang et al. [139] showed that ASIC1 channels promote the growth of gastric cancer by upregulating autophagy. It would be interesting to expand on this study and investigate the potential effects of other ASICs on autophagy in cancer.

TRPM5 encodes a voltage-sensitive monovalent cation-selective channel, which is activated by elevated Ca^{2+} [91, 124]. EA can also influence *TRPM5*-activity [140]. Decreases in extracellular pH either quickly block *TRPM5*-induced current (IC_{50} at pH

6.2) or slowly enhance current inactivation [140]; the former is reversible while the latter is irreversible [140]. Our findings show that high *TRPM5*-expression was associated with improved survival outcomes in OAC. *TRPM5*-expression was upregulated compared to normal tissue in both the UALCAN and OCCAMS datasets. It was also upregulated in various OAC tumor grades and nodal metastatic stages in the OCCAMS dataset only. There are no studies in the literature examining the role of *TRPM5* in OAC. In an mRNA-expression analysis of bladder carcinoma patients, Ceylan et al. [141] reported significant reductions in *TRPM5*-expression in patient tissue. *TRPM5* was hypomethylated in OSCC compared to normal tissue [142]; whether such hypomethylation led to increased *TRPM5*-mRNA expression was not reported. When we examined the UALCAN portal for *TRPM5*-expression in OSCC tumor tissue, we noted no significant difference, compared to normal tissue. In a pan-cancer analysis by Qin et al. [143], no significant differences in *TRPM5* transcripts were observed between normal tissue and breast cancer, lung cancer, and colorectal cancer samples. In a hospital-based case-control study on nucleotide polymorphisms in childhood leukemia, it was observed that patients with the CG or GG genotype of the rs2301696 location in *TRPM5* had a decreased risk of developing childhood leukemia, compared to those with the CC genotype [144]. One study examined the association of *TRPM5* with patient survival in various cancers. In contrast to our findings in OAC, high *TRPM5*-mRNA expression correlated with poor overall survival in patients with melanoma and gastric cancer [50]; high *TRPM5*-expression did not, however, correlate with poor, overall survival in patients with ovarian, lung, breast, or rectal cancer [50]. In agreement with our findings from the OCCAMS dataset, *TRPM5*-expression has been implicated in metastasis [50, 51]. Sutoo et al. [51] noted that the adaptation of lung cancer cells to chronic acidic extracellular conditions (pH 6.2) elicited a sustained increase in lung cancer cell invasion and metastasis, with *TRPM5* being expressed in these cells. Similarly, in murine B16-BL6 melanoma cells, *TRPM5* mediated acidic extracellular-pH signalling, whereas *TRPM5* inhibition reduced spontaneous metastasis in these cells [50].

In the current study, *TRPM5*-upregulation was associated with improved survival outcomes. Literature citing a mechanism underlying the association of *TRPM5* with cancer is scarce. The literature which does exist focuses on *TRPM5*-related mechanisms in the tumor microenvironment (TME), or in cancer metastasis [50, 145, 146]. Mucin, secreted by goblet cells, is needed to form a physical barrier to protect epithelial cells from stress-induced damage (including acid-induced damage). Mitrovic et al. [146] concluded that, in a human colonic cancer goblet cell line, *TRPM5* mediated (via the NCX1) the entry of Na^+ to the cell; this, in turn, resulted in the uptake of Ca^{2+} and the secretion of mucin 5AC. The upregulation of *TRPM5* in OAC tissue in the present study therefore might protect the lining of the oesophagus following exposure to EA. Sakaguchi et al. [145] demonstrated a role for *TRPM5* in immune cells: they showed that *TRPM5* negatively regulated Ca^{2+}-dependent inflammatory responses [production of IL-6 and chemokine C-X-C ligand 10 (CXCL10)] in B lymphocytes. Some authors have already described the role of immune cells in OAC [147-149]. Again, the upregulation of *TRPM5* observed in the present study may protect the oesophagus from deleterious inflammatory responses. Maeda et al. [50] established a pathway for *TRPM5*-mediated, lung metastasis in a murine melanoma cell line: following activation by EA, *TRPM5* increased $[Ca^{2+}]_c$; this, in turn, activated nuclear factor kB (NF-kB) which subsequently increased the expression of matrix metalloproteinase-9. Matrix metalloproteinase-9 potentially supports the lung metastasis in this cell line, by degrading collagen in the extracellular matrix [50]. Further study in human tissue is needed to verify these findings.

ATP2C2 encodes an ATPase pump involved in the transport of Ca^{2+} and Mn^{2+} into the Golgi [92]. It is also involved in Ca^{2+} signalling independent of its ATPase activity [92]. In particular, SPCA2 (the protein encoded by the *ATP2C2* gene) interacts with the SOCE channel, Orai1, and induces Ca^{2+} influx at the cell surface [150]. It has been shown that unbalanced, store-independent Ca^{2+} signalling can lead to enhanced cell proliferation and tumorigenesis [150]. Our findings show that *ATP2C2*-expression was upregulated in OAC in both datasets. In addition, higher *ATP2C2*-expression was associated with improved patient survival. Further analysis revealed that *ATP2C2* was significantly upregulated in various OAC tumor grades and nodal-metastatic stages in

both datasets. *ORAI1* (encoding the Orai1 channel) is upregulated in OAC tissue compared to normal tissue in both the UALCAN and OCCAMS datasets, supporting this potential store-independent interaction [150]. Our *ATP2C2*-expression analysis results are consistent with those of Hyland et al. [151], who analysed gene expression in BO. Comparison of BO-samples to same-patient, normal mucosa from squamous oesophagus revealed a 2.33-fold increase in *ATP2C2*-expression in BO [151]. Similar observations have been made in breast cancer analyses. In one breast cancer study, *ATP2C2* was upregulated compared to normal tissue [152]. In a separate set of breast cancer samples, SPCA2 knockdown enhanced sensitivity to DNA-damaging agents, including doxorubicin, cisplatin, and ionizing radiation [153]. Survival-related observations in the literature contradict our findings. A gene-expression study [152] noted that high *ATP2C2*-expression was associated with worsened patient survival in OSCC, breast cancer, thyroid carcinoma, head-and-neck squamous cell carcinoma, kidney, renal clear cell carcinoma, and lung squamous cell carcinoma [152]. Liu et al. [152] showed that *ATP2C2*-expression negatively correlated with patient survival in breast cancer. In a study by Makena et al. [153] on the SPCA2 protein, high abundance was associated with poor prognosis in luminal ER^+/PR^+ breast cancer subtypes. Similarly, Zhao et al. [154] (TCGA data) revealed a 76%-decreased survival rate among thyroid cancer patients with the *ATP2C2* gene, compared to those without. Again, in contrast to our OAC findings, Zhang et al. [155] noted that the long-non-coding-RNA version of *ATP2C2* (ATP2C2-antisense 1) was associated with worsened overall survival in thyroid carcinoma. Metastasis-related studies show inconsistent results. Similar to our findings, a breast cancer analysis by Liu et al. [152] showed that *ATP2C2*-expression was correlated with advanced breast cancer stages (the "T" and "N" components). A separate, breast cancer study, however, showed that high SPCA2 levels protected against the initiation of the epithelial-mesenchymal transition [156].

ATP2C2 upregulation was associated with improved patient survival in the present study. There is a lack of data in the literature highlighting improved patient survival with *ATP2C2*-upregulation, for any cancer. The literature on carcinogenesis points to either an Orai1-related mechanism, extracellular signal-regulated kinase 1/2 (ERK1/2)-activation, the adaptation to hypoxia, or to the influence of *ATP2C2* on the

TME [92, 152, 157-159]. In OSCC, tumours displayed an increased abundance of the Orai1 protein, and this increased abundance was associated with poor overall and recurrence-free survival; furthermore, pharmacological antagonists of Orai1 reduced OSCC proliferation, invasion, and tumorigenesis [157]. Kohn et al. [158] noted that high SPCA2-abundance was correlated with epithelial genes in cancer cell lines. Similarly, Feng et al. [92] demonstrated that SPCA2-overexpression conferred increased proliferation in a non-malignant mammary, epithelial cell line. The authors demonstrated that this increased proliferation was due to the activation of the ERK1/2 pathway [92]. Jenkins et al. [159] showed that *ATP2C2* helped colon cancer cells adapt to hypoxia, prevented cell death, increased proliferation capacity, and promoted tumor growth. Liu et al. [152] demonstrated that *ATP2C2* might be a potential indicator of TME status. Specifically, genes from the patient group with low *ATP2C2*-expression were significantly enriched in immune-related activities, whereas those from the high, *ATP2C2*-expression group were mainly enriched in metabolic pathways [152]. The *ATP2C2,* low-expression group had increased numbers of pro-tumor M2 macrophages and decreased numbers of anti-tumor M1 macrophages [152]. The immune cell profile of the TME (of breast cancer in the study by Liu et al. [152]) may therefore override the tumorigenic effects inside the cell and warrants further study. Similarly, the acidic TME of OAC warrants further study and may explain improved survival in OAC patients and not in other cancers.

GRIN2D encodes the 2D subunit of the NMDAR. NMDARs are ligand-gated, glutamate receptors involved in Ca^{2+} signalling, primarily in neurons [160]. Our data shows that *GRIN2D*-expression was upregulated in OAC: the gene is the highest weighted-ranking gene in the two datasets examined. Zhang et al. [161] showed that *GRIN2D*-expression was upregulated in 5 OSCC samples from Chinese patients. Conversely, *GRIN2D* was among the 13 genes that were hypermethylated (downregulated) in both OAC and OSCC [162]. In meibomian cell carcinoma (cancer of the glands of the eyelids), *GRIN2D*-expression was also downregulated, compared to normal tissue [163]. In a study of various cancer cell types by Luksch et al. [164], knockdown of *GRIN2D* did not influence phenotype. There are no reported studies demonstrating a role for *GRIN2D* in patient survival. The NMDAR-2D subunit has been shown to play

a role in breast-to-brain metastasis [165]. Endothelial *GRIN2D* has been shown to promote angiogenesis in colorectal cancer [166].

TRPC4 encodes a voltage- and ligand-gated cation channel, which alters enzymatic activity and initiates endocytosis and exocytosis [167]. TRPC4 channel activity is potentiated by decreases in pH [168]. Our data shows that *TRPC4*-expression was upregulated in OAC relative to normal tissue: this gene has the second-highest weighted ranking for expression (TPM) in our study. There is currently no reported evidence linking *TRPC4* with OAC. Zhang et al. [169] found in a study of 2,433 cases and 2,433 controls, that the *TRPC4* polymorphisms rs9547991 and rs978156 were candidate susceptibility markers for lung cancer in a Chinese population. Subjects carrying at least one variant allele had a 1.29-fold increased risk of developing lung cancer, compared with those carrying no variant alleles [169]. By contrast, TRPC4 has been shown to inhibit the proliferation of renal cell carcinoma cells, after the cells were exposed to englerin A (an anti-cancer substance, found in the bark of the *Phyllanthus engleri* tree) [170]. Consistent with our findings, there are no data in the literature to suggest a role for *TRPC4 i*n patient survival**.** *TRPC4*-activation by *GPR68* agonists promoted the invasion and metastasis of granule precursor-derived human medulloblastoma [171]. *TRPC4*-downregulation has been proposed as a trigger for tumor angiogenesis in renal cell carcinoma [172].

TRPM2 encodes a Ca^{2+}-permeable non-selective cation channel which is activated by adenosine diphosphate ribose (ADP-ribose), increased temperature, oxidative stress, and Ca^{2+} [173-175]. TRPM2-gating is inhibited at pH 5.5 to 6.7, indicating a role in acid-sensing [176]. Our data shows that *TRPM2*-expression was upregulated in OAC: the gene has the third-highest weighted rank. There is no evidence linking a role for *TRPM2* in OAC; evidence for a role of *TRPM2* in other cancers is more extensive. A study of TRP-family-mRNA expression by Qin et al.[143], revealed that *TRPM2*-expression was upregulated in breast cancer (ductal carcinoma and invasive breast cancer), small-cell, lung carcinoma, colorectal cancer (colon and caecum adenocarcinoma), gastric cancer, and melanoma. Conversely, *TRPM2*-expression was downregulated in prostate cancer and in the brain and central nervous system

cancers [143]. Sumoza-Toledo et al. [177] noted somewhat similar results in breast cancer: *TRPM2* was upregulated in invasive breast carcinoma, compared to normal tissue. *TRPM2*-expression was upregulated in oral squamous cell carcinoma (OrSCC) tissue [178]. Findings from an *in vitro* study [179] on prostate cancer contradict those from Qin et al.[143]: here, *TRPM2* played a key role in prostate cancer proliferation, as demonstrated by small interfering RNA techniques [179]. In non-small-cell, lung cancer, the long non-coding RNA (TRPM2-antisense RNA (AS)) was upregulated, and subsequent downregulation of *TRPM2* promoted apoptosis *in vitro* [180]. In OrSCC, low *TRPM2*-expression was associated with poorly- or moderately-differentiated, tumor tissue grades [181]. *TRPM2*-expression also predicted survival outcomes in breast, lung, or colorectal cancers [143]. In contrast to Qin et al. [143], a study by Sumoza-Toledo et al. [177] demonstrated that high expression was associated with improved survival outcomes in both the human epidermal growth factor receptor 2 (HER2) and ER, breast cancer subtypes. In contrast to findings reported by Chen et al. [183], Gil-Kluick et al. [183] reported that high *TRPM2*-expression was associated with improved patient survival in acute myeloid leukaemia (AML). Several mechanisms underlying TRPM2 function have been cited. Chen et al. [182] showed that *TRPM2* promoted AML proliferation and cellular survival through the modulation of mitochondrial function, ROS, and autophagy. In both *in vitro* and murine model studies, Almasi et al. [184] suggested that *TRPM2* promoted gastric-cancer migration, invasion, and tumor growth through the AKT pathway; the same group previously demonstrated that *TRPM2* promoted gastric-cancer cell survival via the c-Jun NH2-terminal kinase (JNK) pathway [185]. Two studies examined the role of *TRPM2* in pancreatic ductal carcinoma: the first observed that *TRPM2*-overexpression promoted cell proliferation, invasion, and migration [186]; the second implicated the protein kinase C/mitogen activated protein kinase pathway as the mechanism by which TRPM2 exerts these effects [187]. *TRPM2* was also shown to be essential for the survival and migration of OrSCC [178].

The present study has several limitations. Our analysis is based exclusively on transcriptomic data: this does not account for potential changes in protein function, resulting from altered translational or post-translational mechanisms. Evidence from

an ovarian-cancer xenograft model, however, has indicated that the correlation between mRNA and protein abundance is closer for differentially expressed genes than for those whose expression is not altered [188]. A comparative analysis, using a proteomic dataset, would make the results of the present study more robust. The use of NAT, as a source of non-cancerous cells in UALCAN, is also a potential source of error: these cells might potentially be in a cancerous or pre-cancerous state [189]. Indeed, Aran et al. [189] noted that NAT may have properties distinguishing it from both tumor tissue and a more stringent classification of normal tissue; none of the 18 genes highlighted in the study were investigated in the present study. The statistical power of each dataset has an influence on the results obtained. The UALCAN portal compares data from 89 OAC-tumor samples with 11 NAT samples, while the OCCAMS dataset compares data from 213 samples with 15 normal-tissue samples. We note that none of the 21 survival-associated genes from the OCCAMS dataset were significant in UALCAN, after adjustment. We also note that a higher proportion (67.2%) of OCCAMS, heatmap-related *t*-tests proved significant after adjustment for MOT, than in UALCAN (42.9%). It is possible that the UALCAN portal did not have sufficient power to detect all statistically significant differences.

In conclusion, the present study has implicated 6 genes (*CACNA1D*, *CACNA2D4*, *JPH1*, *ACCN4*, *TRPM5,* and *ATP2C2*) as potential prognostic markers and 3 genes (*GRIN2D*, *TRPC4,* and *TRPM2*) as candidate diagnostic markers for OAC. Of these, *ACCN4*, *TRPM5*, *TRPC4*, and *TRPM2* have established roles in acid-sensing [140, 168, 176, 190]. With the exception of *CACNA1D* (OAC-expression data), *ATP2C2* (BO-expression data), and *GRIN2D* (OAC-methylation data), published, OAC-related data is lacking for the genes highlighted by the current study. Higher expression of all 6 prognostic genes in this study was associated with improved survival outcomes. These positive survival observations were noted in conjunction with high expression of all of these 6 genes in OAC tissue, compared to normal tissue, and sometimes (as in the case of *CACNA1D*, *JPH1*, and *ATP2C2*) in advanced metastatic stages and tumor grades. We hypothesize that these genes may be either increasing tumor cell death and/or inhibiting other cancer hallmarks, either directly or by indirect mechanisms. In the literature, the association of *CACNA1D* with improved survival outcomes in colon

adenocarcinoma, and of *JPH1* with improved survival outcomes in small-cell lung carcinoma, was particularly strong [108, 127]. The associations of the other genes with survival outcomes in the literature were either inconsistent with those of our study (*CACNA2D4*, *TRPM5*, and *ATP2C2*) or unreported (*ACCN4*). With the exception of *JPH1* (targeted by miR-145 to favour tumor-suppression), *CACNA2D4* (mitigation of the adverse effects of chemotherapy in BRD9, gastric cancer), and *TRPM5* (the Ca^{2+}-dependent regulation of inflammatory responses and the production of mucin), there are no clear molecular mechanisms cited to explain the improved patient survival observations in the present study. Indeed, most mechanisms cited in other cancers would intuitively lead to worsened patient survival. Because an acidic environment is what differentiates OAC from many cancers, the potential role of EA in OAC is again brought into focus. Three studies have implicated *TRPM2* in metastasis. Other studies also consistently linked high expression of *CACNA1D*, *CACNA2D4*, *JPH1*, *TRPM5*, *ATP2C2*, *GRIN2D*, and *TRPC4* with metastasis. Further research is required to discern which of these 9 genes could prove useful in OAC prognosis or diagnosis. Given the ubiquitous expression of *GRIN2D* in OAC and OSCC, *TRPC4* and *TRPM2* may be more selective, diagnostic markers for OAC. The potential interaction between the following partners in OAC may yield promising results: *CACNA1D* with Ca_v subunits, the RYR1 and the G protein-coupled oestrogen receptor; *JPH1* and miR-145; *ACCN4* and *GPR65*; *TRPM5* and *NCX*s; *ATP2C2* and *ORAI1*; and *TRPC4* and *GPR68*. The role of the TME (particularly acidic and immune components) should also be considered in the design of such experiments. Taken together, the present study lays the groundwork for further exploration of potentially oncogenic and protective Ca^{2+}-signalling pathways in OAC.

6. References

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CHAPTER THREE: The role of bile acids in the development of Barrett's oesophagus and oesophageal adenocarcinoma: a systematic review

This chapter is based on research carried out in 2022. A manuscript has been written up (below), but is currently not under consideration by any journal.

The role of bile acids in the development of Barrett's oesophagus and oesophageal adenocarcinoma: a systematic review

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1. Abstract

Background

Oesophageal adenocarcinoma (OAC) and its precursor, Barrett's oesophagus (BO), have
overlapping risk factors, including gastro-oesophageal reflux disease. Refluxed contents
contain bile acids (BAs) in an acidic environment. Exposure to BAs in acidic environment has been associated with BO and OAC development; however, little research has been carried out on the effects of BAs at a neutral pH.

Aims

This study explores the potential associations between neutral pH, BA exposure and development of OAC and BO, and aims to identify whether individual BAs may contribute to the underlying biology of these conditions.

Methods

A systematic review of 6 computerised databases was conducted on original articles involving oesophageal tissue from human subjects or oesophageal cell-lines. All articles retrieved for inclusion examined effects of BAs, at neutral pH, on development or risk reduction of BO or OAC.

Results

Key observations from the 20 studies included were that deoxycholic acid exerted effects on BA-induced BO and OAC through several potentially co-operating mechanisms, including oxidative stress, DNA damage, inflammation, proliferation, apoptosis, apoptotic resistance and angiogenesis. In BO, taurodeoxycholic acid was associated with oxidative-stress and DNA damage. Treatment of non-malignant human oesophageal cells and BO cells with ursodeoxycholic acid in vitro resulted in a reduction of deoxycholic-acid-induced inflammation.

Conclusions

In conclusion, exposure to BAs at a neutral pH is associated with biological features linked to cancer development, which could be targeted therapeutically, through medication, bacterial supplementation, or lifestyle modifications.

Keywords: Oesophageal Adenocarcinoma, Barrett's Oesophagus, bile acids, deoxycholic acid, taurodeoxycholic acid, ursodeoxycholic acid.

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Declarations

Funding

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Conflict of Interest

The authors have no conflicts of interest to declare.

Data Availability Statement

All data procured during the production of this systematic review has been presented in tabular form in the present study. The keywords used in our database searches are available in Supplemental Material. All ethics declarations have been made in the individual studies.

Code Availability

No code was produced in this systematic review process.

Author Contributions

ALC designed the protocol for the present systematic review. She input the keywords into the six databases, checked reference lists of the papers generated, compiled the shortlisted papers in Rayyan (https://www.rayyan.ai/), screened the shortlisted papers for eligibility and synthesised relevant information from the papers in six separate tables. She also prepared all drafts of the paper. ARV input keywords into the six databases, compiled the shortlisted papers in Rayyan, screened the shortlisted papers for eligibility and synthesised relevant information from the papers in six separate tables: information which was checked against ALC's observations.

SLMCK reviewed drafts of this article and provided feedback.

FJD reviewed drafts of this article and provided feedback.

JJM provided the hypothesis for the study, provided input at all stages of the research and reviewed drafts of the article, providing feedback.

2. Introduction

Oesophageal adenocarcinoma, OAC, is a cancer of the oesophagus formed from the conversion of non-malignant squamous cells to glandular cancerous cells [3]. A precursor of OAC, Barrett's oesophagus, (BO), also has a glandular phenotype [223].

Patients with BO and OAC often have gastro-oesophageal reflux disease, (GORD), where the contents of the stomach and duodenum can flow back into the lower oesophagus [33], [224]. This refluxate contains bile acids, (BAs), mixed in an environment of hydrochloric acid, (HCl), and other gastric secretions [33], [225]. Exposure of oesophageal cells and tissue to this lower pH mixture has been linked to the development of BO and OAC [226], [227]. Whether BAs act independently of acid-induced damage has not been fully elucidated. Given that BO and OAC persist despite the prescribing of acid-suppressing and acid-neutralizing medication [228], [229], the role of BA exposure independent of its acidic environment cannot be ignored. As GORD progresses to BO, and BO progresses to OAC, the proportion of HCl and secretions in the refluxate decreases dramatically, leaving predominantly BAs [199], [226], [230]. Such an observation highlights their clinical relevance. There has been no recent systematic study on whether BAs have an effect on cancer-associated processes in the oesophagus independently of an acidic environment. Such a study may identify patients at most risk to develop BO or OAC and may offer more tailored treatment for such patients.

Oesophageal malignancies are the sixth-leading cause of cancer-related mortality worldwide [1], [4]. OAC is the most common form in Northern Europe (incidence of 3.3 per 100,000 persons) and Western Europe (1.8 per 100,000 persons) [11]. OAC is the most common type of oesophageal cancer in Ireland, accounting for 50-90% of all OACs diagnosed from 2010 to 2014 [10]. European and North American data also indicate that OAC is nine times more common in men than in women [231]. In most populations, OAC has an overall five-year survival rate of under 15% [14], [15]. The incidence of OAC is on the rise [3], [232]. For example, the incidence of OAC in England has increased more than sixfold in the last 30 years [3]. It is also predicted that new OAC cases will rise by 82% in the 19-year period from 2021 to 2040 [13].

BAs are cholesterol derivatives that exert both metabolic and hormonal effects on the human body [187], [233]. They are components of bile, whose functions include emulsification of lipids (to aid absorption in the small intestine), absorption of fat-soluble vitamins, metabolism and excretion of cholesterol, excretion of bilirubin,

and the dissolution of gallstones [187], [233]. There are both primary (synthesized in the liver) and secondary (synthesized in the small intestine) BAs [187], [233]. The primary BA cholic acid (CA) is converted to either deoxycholic acid (DCA) or ursodeoxycholic acid (UDCA); whereas chenodeoxycholic acid (CDCA), the other primary BA, is converted to lithocholic acid (LCA) or UDCA [187], [233]. These conversions are executed through the action of bacterial dehydroxylating enzymes in the small intestine. The conversion of primary to secondary BAs is depicted in Figures 3.1 and 3.2.

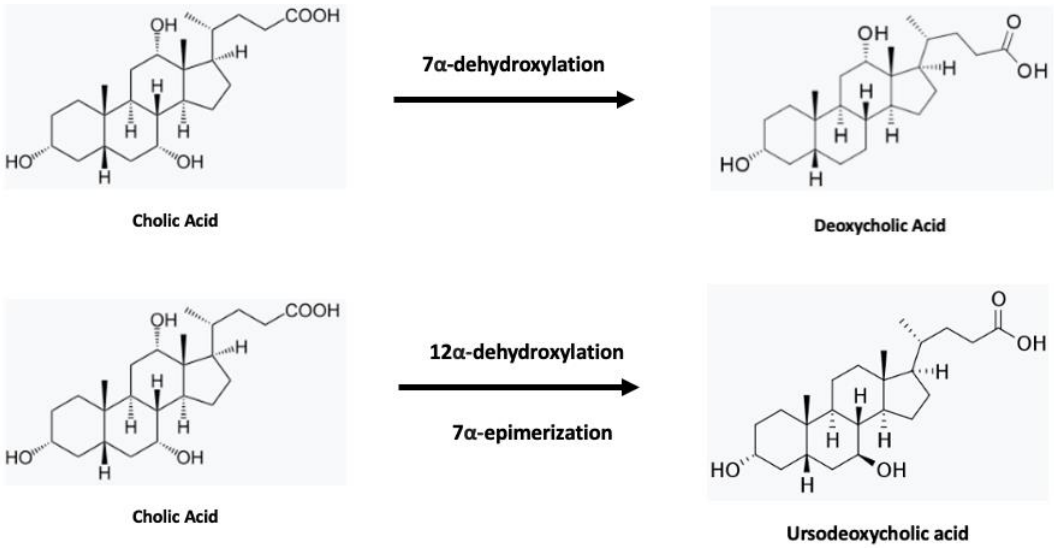

Figure 3.1. Diagram depicting the conversion of Cholic Acid (CA) to Deoxycholic Acid (DCA) and Ursodeoxycholic Acid (UDCA)

7α-dehydroxylation is the removal of a hydroxyl (OH) group from carbon seven (alpha configuration) of the bile acid. 12α-dehydroxylation is the removal of a hydroxyl (OH) group from carbon twelve (alpha configuration) of the bile acid and 7α-epimerization is the isomerization of the hydroxyl (OH) group from carbon seven from an alpha to a beta position.

Source: van Mil SW, Houwen RH, Klomp LW. Genetics of familial intrahepatic cholestasis syndromes. J Med Genet. 2005 Jun;42(6):449-63. doi: 10.1136/jmg.2004.026187. PMID: 15937079; PMCID: PMC1736078

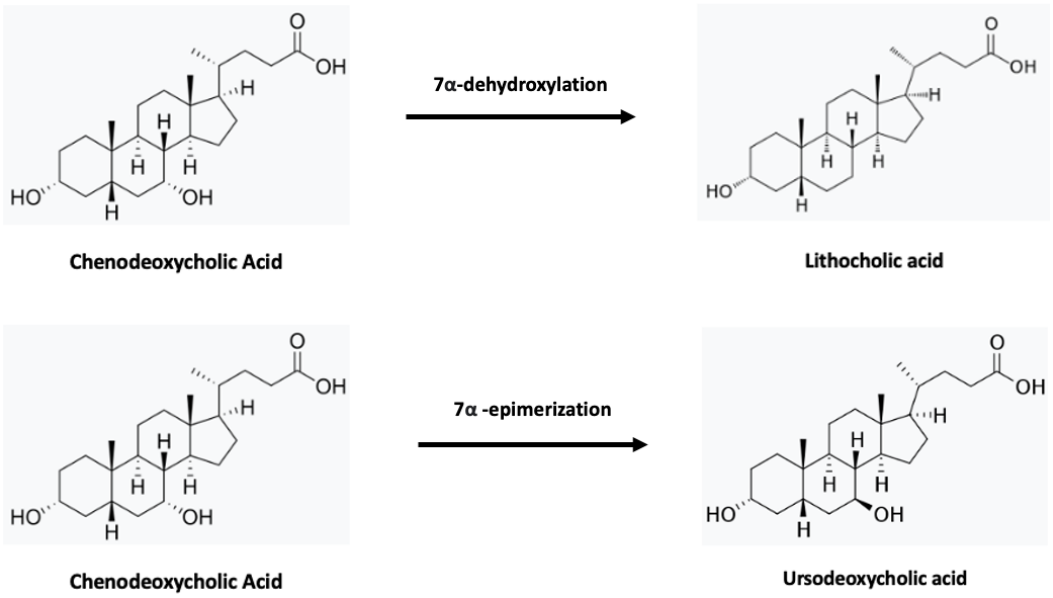

Figure 3.2. Diagram depicting the conversion of Chenodeoxycholic Acid (CDCA) to Lithocholic Acid (LCA) and Ursodeoxycholic Acid (UDCA)

7α-dehydroxylation is the removal of a hydroxyl (OH) group from carbon seven (alpha configuration) of the bile acid. 7α-epimerization is the isomerization of the hydroxyl (OH) group from carbon seven from an alpha to a beta position

Source: van Mil SW, Houwen RH, Klomp LW. Genetics of familial intrahepatic cholestasis syndromes. J Med Genet. 2005 Jun;42(6):449-63. doi: 10.1136/jmg.2004.026187. PMID: 15937079; PMCID: PMC1736078

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BAs exist in both unconjugated and conjugated forms [187], [188]. Conjugated BAs, conjugated to the amino acids glycine or taurine, are the most predominant type in bile, accounting for >90% of total BAs in the bile of healthy individuals [187], [189], [234]. Conjugated BAs, mainly glycocholic acid (GCA, 60% of total BA pool) and

other glycine conjugates, are the main constituents of the GERD refluxate [235]. The secondary BA DCA and the conjugated BA TDCA are the most common BAs in the BO refluxate [199]. The profile of BAs in the OAC refluxate also contains a higher proportion of conjugated BAs compared to that of GORD patients [234]. Conjugated BA levels in the refluxate from patients with advanced BO or OAC are significantly higher than from patients with benign BO [199]. Furthermore, conjugated BAs such as TCA or TDCA were reported to increase proliferation of OAC cell lines [236]. Hydrophobic BAs, including GCA, LCA, and DCA, are considered to be the most toxic BAs given that they are a major factor in inducing liver cell death [190]. The hydrophilic BAs, including CA, CDCA, and UDCA, are reported to be cancer-protective [190]. The toxic effects of BAs vary depending on the pH of the environment, the concentration of the BAs in question, and the duration of BA exposure to the cells [224], [237].

Bile is produced in the liver, stored in the gallbladder, and released into the small intestine in response to dietary fat [187], [238]. 95% of the BAs that enter the intestine are recirculated back to the liver and recycled, a process known as enterohepatic recirculation [238]. When the sphincters at the end of the stomach fail to close properly, acid from the stomach and BAs from the duodenum can reflux into the oesophagus [224]. The lower oesophageal sphincter and the pyloric sphincter can fail to close for many reasons: stagnant food in the stomach (increasing pressure); central obesity (increasing pressure on the stomach); sliding hiatal hernia (where the stomach intermittently slides into the chest); a peptic ulcer (blocking full sphincter closure); a sleeve gastrectomy (which increases pressure in the remaining stomach); and reduced lower oesophageal sphincter pressure (loosening the sphincter) [224], [239]. Removal of the gallbladder leads to excess bile in the duodenum and overflow into the stomach [224]. Finally, it has been shown that BA exposure relaxes the lower oesophageal sphincter through interaction with the BA receptor Takeda G protein-coupled receptor 5 (TGR5) [240].

Several biological processes underlying cancer development have been linked to BAs [193], [194], [195], [196], [197], [198]. These include oxidative stress, DNA

damage, inflammation, cell proliferation, resistance to apoptosis, and angiogenesis [193], [194], [195], [196], [197], [198]. It is most likely that these BA-induced effects are mediated by BA receptors: farnesoid X receptor (FXR), TGR5, vitamin D receptor (VDR), sphingosine 1 phosphate receptor 2 (S1PR2), and pregnane X receptor (PXR) [210], [220], [241], [242], [243]. Each of these receptors has its own homeostatic functions [202], [215], [219], [242], [244], [245], but has also been associated with certain cancers, including OAC and BO [200], [202], [210], [244], [246], [247]. Their affinity for individual BAs also differs [201], [213]. The biological switch of BA receptor function from being cancer-protective to cancer-promoting in the oesophagus most likely depends on BA concentration, the duration of BA exposure, and the disease state of the oesophagus [191], [192], [214], [248].

Two relatively recent systematic reviews on BA-exposure examined the effects of acidic environments alone, BAs at a neutral pH and a combination of BAs in acidic environments [188], [191]. McQuaid *et al*. examined 83 original articles on human participants, human oesophageal tissue or human cell-lines [191]. Their study outcomes included oesophagitis, BO and OAC and any underlying mechanisms [191]. Bus *et al.* examined 6 cell-line studies with BO and OAC being primary outcomes [188]. The authors of each review concluded, amidst variation in study designs, that BAs (including those independent of acidic environments) may contribute to the symptoms of oesophagitis, BO and OAC [188], [191]. There is thus a need for an update on the topic, with an expanded focus on the effects of BAs at a neutral pH and on OAC development.

In the current study, we carried out a systematic review of research papers covering the topic of neutral pH BA-exposure on the development or risk of BO or OAC. Such a review was carried out on human subject, human tissue and human cell-line studies only. Specifically, we aimed to explore the potential association between neutral pH BA exposure and the biology of OAC and BO; to identify the individual BAs to whose exposure altered OAC and BO biology; and to identify key proteins involved in BA-dependent development of BO and OAC.

3. **Methods**

Search Strategy

We searched PubMed, EMBASE, CINAHL, Cochrane Library, Scopus and Web of Science for articles addressing our study aims. We limited our search to papers published from the 1st January 2013 to 31st December 2022. Details of keywords used have been included in Supplemental Table S1. In brief, we searched keywords for the following concepts: OAC, BO, BAs, cancer-biology outcomes and study design. Only original articles were included. We did not search for abstracts or unpublished data. There were no language restrictions. Any non-English papers were translated using Google Translate (https://translate.google.com).

Full articles were retrieved for the abstracts meeting the inclusion criteria. A search of the bibliographies of included articles was also carried out to retrieve articles potentially missed by the initial search strategy.

Study Inclusion and Exclusion Criteria

Table 3.1. details the inclusion and exclusion criteria for human studies included in the present systematic review. Table 3.2. details the inclusion and exclusion criteria for *in vitro* studies in the present systematic review

Table 3.1. Inclusion and Exclusion Criteria for Human Studies included in the Present Systematic Review

INCLUSION CRITERIA	**EXCLUSION CRITERIA**
Oesophageal Adenocarcinoma Patients	Oesophageal Squamous Cell Carcinoma Patients
Barrett's Oesophagus Patients	Oesophageal Adenocarcinoma Patients with secondary cancers
Male and female patients*	Patients receiving chemotherapy or radiation
Patients aged 18 or over	
Exposure to individual bile acids	Acid exposure alone

Exposure to a mixture of bile acids	Exposure to acid and bile acids as a mixture
Randomised controlled trials	Studies measuring surrogate markers of oesophageal bile reflux**
Cohort studies (prospective and retrospective)	Narrative reviews
Case-control studies	Cross-sectional studies
Studies on *in vivo* experimentation on human oesophageal tissue	Murine studies
	Cancer migration
	Cancer invasion
	Metastasis-related outcomes
	Patients taking medications targeting stomach acid or bile acids***

* There were no restrictions on patient ethnicity, patient socio-economic class or geographical region in which the studies took place

**Alkaline pH or bilirubin

*** Proton pump inhibitors, BA sequestrants, antacids, H-2 blockers, alginates and prokinetics

Table 3.2. Inclusion and Exclusion Criteria for *In Vitro* Studies in the Present Systematic Review	
INCLUSION CRITERIA	**EXCLUSION CRITERIA**
Oesophageal Adenocarcinoma Cell-lines (male and female)	Oesophageal Squamous Cell Carcinoma Cell-lines
Barrett's Oesophagus Cell-lines (male and female)	Non-human cell-lines

Non-malignant, human oesophageal cell-lines	Human non-oesophageal cell-lines
Exposure to individual bile acids	Exposure to acid alone
Exposure to bile acids as a mixture	Exposure to a mixture of acid and bile acids
	Exposure to acid and bile acid treatments*
	Cancer migration
	Cancer invasion
	Metastasis-related outcomes
	Exposure to chemotherapy and radiation
	Narrative reviews
	Murine studies

* Proton pump inhibitors, bile acid sequestrants, antacids, H-2 blockers, alginates and prokinetics

Data Abstraction and Validity Assessment

A data abstraction form was developed prior to full article retrieval, tested by one author on several known articles and revised to improve data recording. All articles meeting the inclusion criteria were reviewed independently by two authors and the data was entered into the abstraction form. We used the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) to assess human study quality [249]. We used the Quality Assessment Tool For In Vitro Studies (QUIN) to assess risk of bias in *in vitro* studies [250]. We did not perform formal quality assessment of the *in vivo* studies.

A protocol for the present systematic review was registered on PROSPERO (ID: 398556).

4. **Results**

Our initial search identified 565 unique titles. This number was reduced to 416 after duplicates were removed. Three hundred and seventy-three articles were excluded for the following reasons: not an original article (62 articles); murine or rat studies (19 articles); study on BAs and acid as a mixture (46 articles); no mention of BA alone (139 articles); no relevant outcomes provided (94 articles); and articles exclusively focusing on cell migration, invasion and metastasis (13 articles). After initial title and abstract review, by two authors, 43 complete articles were considered potentially relevant and retrieved for full review. Twenty-two abstract-only articles were excluded (full texts not retrieved). The final number of articles included in the review was 20, see Figure 3.3.

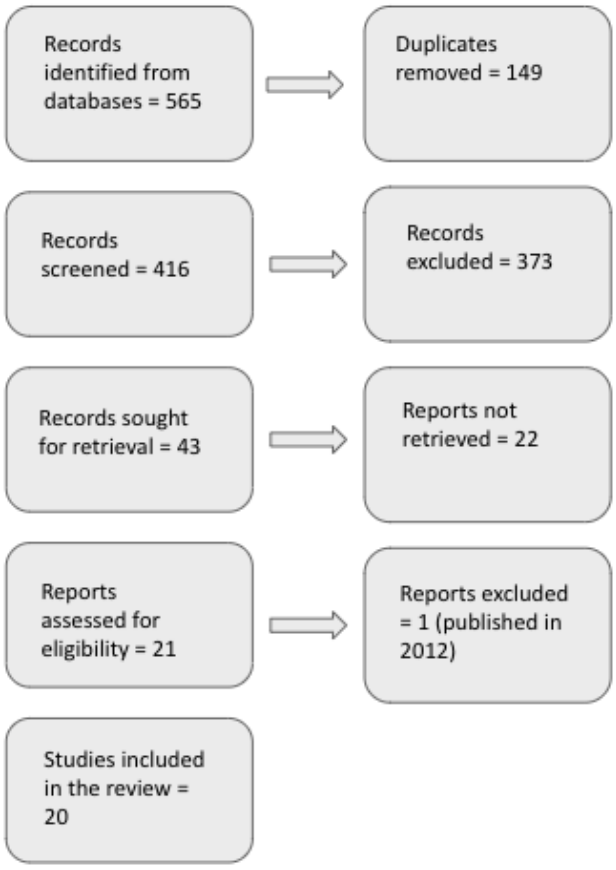

Figure 3.3. PRISMA Flow Chart depicting the Process for Final Article Retrieval

Summary of BA-related Associations with BO and OAC Biology

A summary of the BA-related associations with BO and OAC biology is presented in Figures 3.4 and 3.5, respectively.

In BO studies, DCA was associated with an increase in oxidative stress, DNA damage, inflammation, proliferation, apoptosis, apoptotic resistance and angiogenesis [226], [227], [251], [252], [253], [254], [255]; TDCA was associated with increased oxidative stress and DNA damage [209]; UDCA was associated with a decrease in inflammation; UDCA was associated with unchanged proliferation and apoptosis [226]; and UDCA was associated with both a decrease in DNA damage in one study and had no association in another study [226], [256], Figure 3.4.

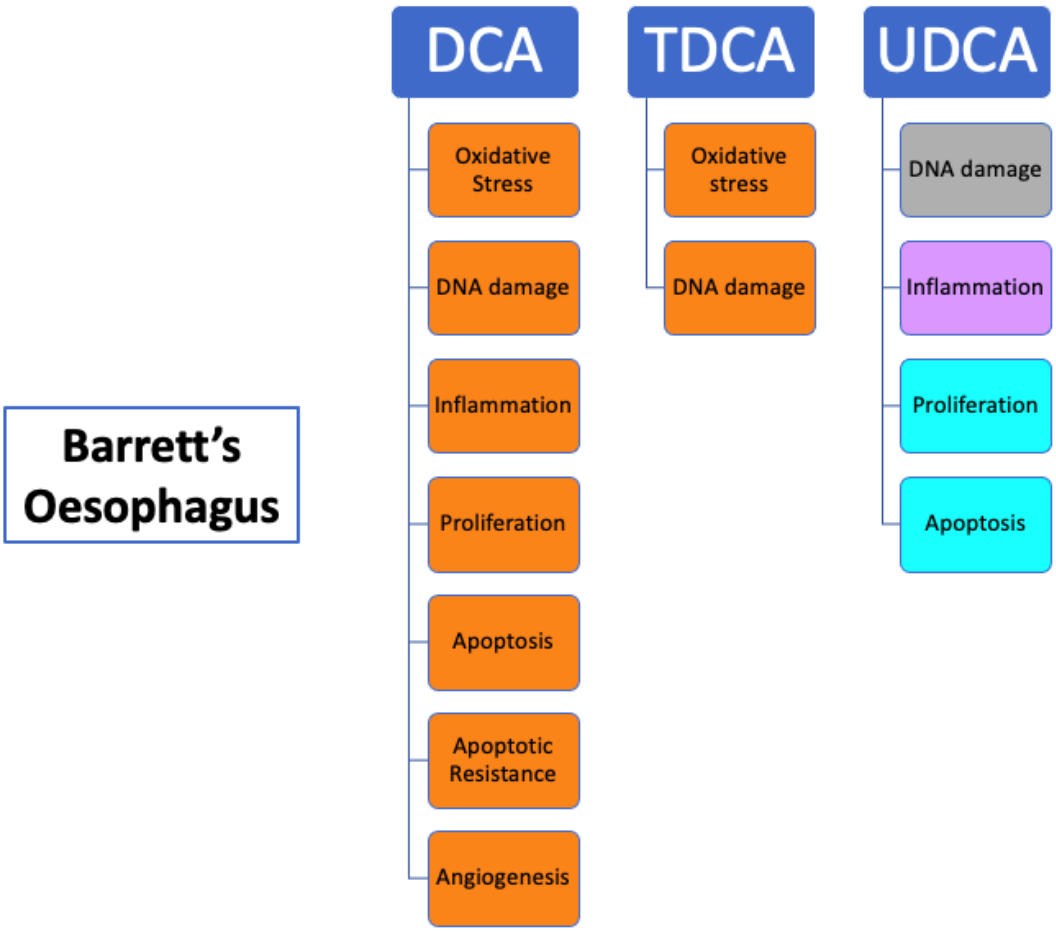

Figure 3.4. Summary of the associations between individual bile acid exposure and changes in tissue biology, leading to Barrett's Oesophagus

Orange, an increase in biological mechanism; purple, a decrease in biological mechanism; blue, no change in biological mechanism; grey, evidence for both a decrease in biological mechanism and no change in biological mechanism.

DCA, deoxycholic acid; TDCA, taurodeoxycholic acid; UDCA, ursodeoxycholic acid

In OAC studies, DCA was associated with an increase in inflammation and proliferation [257], [258]; TCA was associated with an increase in proliferation [203], [259]; CA was associated with an increase in proliferation and apoptosis [260]; and CDCA was associated with an increase in proliferation and apoptosis [260], Figure 3.5.

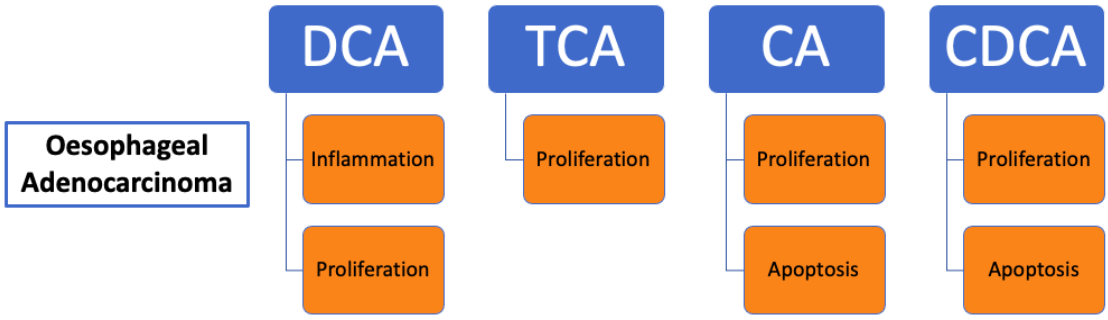

Figure 3.5. Summary of the associations between individual bile acid exposure and changes in tissue biology, leading to Oesophageal Adenocarcinoma

Orange, an increase in biological mechanism

DCA, deoxycholic acid; TCA, taurocholic acid; CA, cholic acid; CDCA, chenodeoxycholic acid

Publications relating to Oxidative Stress

Three studies reported observations relating to oxidative stress [209], [251], [252], see Table 3.3. All of the studies were on human cell-lines and all focused on BO [209], [251], [252]. Two of the three studies examined the effect of DCA on oxidative stress [251], [252], while the other investigated the effect of TDCA [209]. Two studies used ROS production as a molecular marker [209], [251], whereas one

examined delta-like canonical notch ligand 1 (DLL-1, a surrogate marker for oxidative stress) [252]. DCA increased ROS production in one study [251]. DCA increased DLL-1 in another study [252]. The other BA examined, TDCA, also increased ROS production [209]

Table 3.3. Bile-Acid Exposure and Oxidative Stress in Barrett's Oesophagus Studies							
Authors	**Year**	**Study Type**	**Condition**	**Bile Acid(s)**	**Outcome**	**Molecule(s)**	**Result(s)**
Feng et al. [251]	2017	Cell	BO	DCA	Oxidative stress	ROS	DCA induced ROS production in a dose-dependent manner
Tamagawa et al. [252]	2016	Cell	BO	DCA	Oxidative stress	DLL-1	DCA exposure increased DLL-1 production
Li et al. [209]	2016	Cell	BO	TDCA	Oxidative stress	ROS	TDCA induced oxidative stress

Table 3.3. Bile-Acid Exposure and Oxidative Stress in Barrett's Oesophagus Studies

BO = Barrett's Oesophagus; DCA = deoxycholic acid; TDCA = taurodeoxycholic acid; ROS = reactive oxygen species; DLL-1 = delta-like protein 1

Publications relating to DNA Damage

Six studies reported observations relating to DNA damage [209], [226], [227], [253], [256], [261], see Table 3.4. Three studies were carried out in human tissue [226], [256], [261], 2 on human cell-lines [209], [253] and 1 examined the effects of BAs on both human tissue and cell-lines [227]. Two of these studies focused on non-malignant oesophageal cells [227], [261] while 4 focused on BO [209], [226], [253], [256]. Three studies examined the effects of DCA on DNA damage [226], [227], [253] whereas the effects of UDCA, TDCA, a TCA plus GCA mixture, UDCA and DCA (separate experiments) were examined in 1 study each [209], [226], [256], [261].

In non-malignant oesophageal cells, a mixture of TCA plus GCA upregulated phospho-H2A histone family member X (p-H2AX), a marker of DNA damage [261]. Exposure of preneoplastic, hTERT-immortalized Barrett's cells, CP-C and CP-A, to deoxycholic acid (DCA), was reported to induce Minority MOMP (Mitochondrial Outer Membrane Permeabilization), which was associated with upregulation of ROS and DNA damage [253]. Li *et al*. demonstrated, in BO, that TDCA exposure induced DNA damage [209]. Oral UDCA increased glutathione peroxidase 1 (GPX1) levels and prevented DNA-induced DNA damage in BO [226]. 8-hydroxydeoxyguanosine (8OHdG) is an RNA nucleoside which is an oxidative derivative of guanosine [262]. Levels of 8OHdG is used as a biomarker of oxidative stress causing RNA damage [262]. Exposure to UDCA did not change 8OHdG levels in BO [256].

Table 3.4. Bile-Acid Exposure and DNA Damage in Experiments on Non-Malignant Oesophageal Cells or Tissue and Barrett's Oesophagus

NON-MALIGNANT OESOPHAGEAL CELLS OR TISSUE							
Authors	**Year**	**Study Type**	**Bile Acid(s)**	**Condition**	**Outcome**	**Molecule(s) Examined**	**Result(s)**
Wang et al. [227]	2018	Human and cell	DCA	Non-malignant oesophageal cells	DNA damage	Notch 1 and K13	DCA suppressed Notch 1 and K13
Jiang et al. [261]	2016	Human	TCA + GCA mixture	Non-malignant oesophageal tissue	DNA damage	p-H2AX	TCA + GCA mixture upregulated p-H2AX
BARRETT'S OESOPHAGUS							
Xu et al. [253]	2020	Cell	DCA	BO	DNA damage	Cytochrome C and caspase 3	DCA promoted minority MOMP. This resulted in DNA damage
Peng et al. [226]	2014	Human	DCA	BO	DNA damage	p-H2AX and phospho-p65	Oesophageal perfusion with DCA increased p-H2AX and phospho-p65
Li et al. [209]	2016	Cell	TDCA	BO	DNA damage	NOX5-S	TDCA induced DNA damage

Peng et al. [226]	2014	Human	UDCA and DCA (separate experiments)	BO	DNA damage	GPX1	Oral UDCA increased GPX1 levels. Oral UDCA prevented DCA-induced DNA damage
Banerjee et al. [256]	2016	Human	UDCA	BO	DNA damage	8OHdG	Exposure to UDCA did not change 8OHdG levels

Table 3.4. Bile-Acid Exposure and DNA Damage in Experiments on Non-Malignant Oesophageal Cells or Tissue and Barrett's Oesophagus

DCA = deoxycholic acid; TCA = taurocholic acid; GCA = glycocholic acid; p-H2AX = phospho Histone Family, Member X; BO = Barrett's Oesophagus; Minority MOMP = Minority Mitochondrial Outer Membrane Permeabilization; TDCA = taurodeoxycholic acid; NOX5-S = NADPH Oxidase 5; UDCA = Ursodeoxycholic acid; GPX1 = Glutathione Peroxidase 1; 8OHdG = 8-Hydroxydeoxyguanosine

Publications relating to Inflammation

Eight studies focused on the effects of BAs on inflammation [226], [227], [254], [257], [263], [264], [265], [266], see Table 3.5. Five studies were on human cell-lines [226], [257], [263], [265], [266] and the remaining 3 were carried out on human tissue [227], [254], [264]. Four studies were carried out on non-malignant oesophageal cells [263], [264], [265], [266], while others focused on BO (n=3) [226], [227], [254] and OAC (n=1) [257]. Most of the studies investigated the effects of DCA alone (n=4) [227], [254], [257], [265]. The effects of UDCA and DCA (separate experiments) were examined in two studies [226], [266]. One study investigated the effects of TCA, GCA, TDCA and TDCA (all separate experiments within the one study), while another investigated the effects of TCDCA [263], [264].

In non-malignant cell-lines, DCA increased lipid droplets, cyclo-oxygenase 2 (COX-2) and CXC motif chemokine ligand 8 (CXCL-8) expression and interleukin-8 (IL-8) secretion [265]. Another study in non-malignant oesophageal cells noted that DCA-induced production of interkeukin-6 (IL-6) and IL-8 was attenuated by UDCA [266]. TCDCA treatment identified multiple gene sets relating to inflammation in non-malignant cell-lines [264]. Finally, in a study by Shan *et al*., none of the conjugated BAs examined (TCA, GCA, TDCA and TCDCA, all separate experiments) induced IL-8 production in non-malignant oesophageal cells [263].

Four studies have been carried out on the effect of DCA on inflammatory markers in BO (3 studies) and OAC (1 study) [226], [227], [254], [257]. Peng *et al*. demonstrated that, in BO, DCA activated the inflammatory markers, phospho-p65 and nuclear factor kappa b (NF-κB) [226]. By contrast, UDCA upregulated levels of the anti-inflammatory GPX1 and catalase enzymes in BO [226]. DCA also induced expression of COX-2 and prostaglandin E2 (PGE2) in BO [254]; increased mucin 2 (MUC2) expression in BO [227]; and activated COX-2 and NF-κB inhibitor alpha (IκBα) expression in OAC [257].

Table 3.5. Bile-Acid Exposure and Inflammation in Experiments on Non-Malignant Oesophageal Cells or Tissue, Barrett's Oesophagus and Oesophageal Adenocarcinoma							
NON-MALIGNANT OESOPHAGEAL CELLS OR TISSUE							
Authors	**Year**	**Study Type**	**Bile Acid(s)**	**Condition**	**Outcome**	**Molecule(s)**	**Result(s)**
Carrossini et al. [265]	2021	Cell	DCA	Non-malignant oesophageal cells	Inflammation	COX2, CXCL and IL-8	DCA increased LD, COX-2 and CXCL-8 expression and IL-8 secretion
Quilty et al. [266]	2021	Cell	DCA and UDCA (separate experiments)	Non-malignant oesophageal cells	Inflammation	IL-6 and IL-8	DCA-induced production of IL-6 and IL-8 was attenuated by UDCA
Green et al. [264]	2014	Human	TCDCA	Non-malignant oesophageal cells	Inflammation	NF-κB	TCDCA treatment identified multiple gene sets related to inflammation
Shan et al. [263]	2013	Cell	TCA, GCA, TDCA, TCDCA (all separate experiments)	Non-malignant oesophageal cells	Inflammation	IL-8	None of the conjugated BAs, under a neutral condition, induced IL-8 production
BARRETT'S OESOPHAGUS							

Peng et al. [226]	2014	Cell	UDCA and DCA (separate experiments)	BO	Inflammation	NF-κB, GPX1, catalase.	DCA activated NF-κB. Oral UDCA increased GPX1 and catalase levels
Taddei et al. [254]	2014	Human	DCA	BO	Inflammation	COX-2, PGE2	DCA induced expression of COX-2 and PGE2
Wang et al. [227]	2018	Human	DCA	BO	Inflammation	MUC2	DCA increased MUC2 expression
OESOPHAGEAL ADENOCARCINOMA							
Yamada et al. [257]	2014	Cell	DCA	OAC	Inflammation	COX-2 and IκBα	DCA activated COX-2 and IκBα expression

Table 3.5. Bile-Acid Exposure and Inflammation in Experiments on Non-Malignant Oesophageal Cells or Tissue, Barrett's Oesophagus and Oesophageal Adenocarcinoma

DCA = deoxycholic acid; COX-2 = cyclooxygenase 2; CXCL1 = chemokine (C-X-C motif) ligand 1; IL-8 = interleukin 8; LD = lipid droplets; UDCA = ursodeoxycholic acid; IL-6 = interleukin 6; TDCA = taurodeoxycholic acid; NF- κB = Nuclear factor kappa-light-chain-enhancer of activated B cells; TCA = taurocholic acid; GCA = glycocholic acid; TCDCA = taurochenodeoxycholic acid; BO = Barrett's Oesophagus; IκBα = nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; GPX1 = glutathione peroxidase 1; PGE2 = prostaglandin E2; MUC2 = mucin 2; OAC = oesophageal adenocarcinoma

Publications relating to Cell Proliferation

Eleven studies examined the effect of BAs on proliferation [203], [227], [254], [255], [256], [257], [258], [259], [260], [264], [267], see Tables 3.6.A. and 3.6.B. Four were human studies [254], [255], [256], [264] and 7 were carried out in human cell-lines [203], [227], [257], [258], [259], [260], [267]. Two studies were carried out on non-malignant oesophageal cells [264], [267], 4 were carried out on BO [227], [254], [255], [256] and 5 on OAC [203], [257], [258], [259], [260]. The effects of DCA were examined in 5 studies [227], [254], [255], [257], [258]. Two studies were carried out on the effects of TCA and 1 on the effect of TDCA [203], [259], [264]. One study each examined the role of UDCA, CA and CDCA (separate experiments) and a mixture of DCA plus GCA and TCDCA [256], [260], [267].

In non-malignant oesophageal cells, TDCA did not enrich proliferation markers [264]. In a micro-RNA study on non-malignant oesophageal cells, exposure to a mixture of BAs (DCA plus GCA and TCDCA) did not significantly change levels of miR-143, -145 and -192 [267]. In 1 BO study, DCA increased phosphorylated p38 mitogen-activated protein kinase (phospho-p38-MAPK): a molecular associated with cellular proliferation [255]. DCA suppressed Notch 1 activity in BO [227]. UDCA did not change Ki67 levels in BO [256]. DCA induced proliferation via the induction of Vascular Endothelial Growth Factor (VEGF) [254]. In OAC studies, DCA activated

caudal type homeobox 2 (CDX2), Krüppel-like Factor 4 (KLF4) and octamer-binding transcription factor 4 (OCT4) [257], [258]. In other OAC studies, TCA promoted cell proliferation markers, including S1PR2 [203], [259]. CA or CDCA exposure increased levels of miR-221 and -222 in OAC [260]. CA and CDCA exposure degraded CDX2 in OAC [260].

Table 3.6.A. Bile-Acid Exposure and Proliferation Experiments in Non-Malignant Oesophageal Cells and Barrett's Oesophagus							
NON-MALIGNANT OESOPHAGEAL CELLS							
Authors	**Year**	**Study Type**	**Bile Acid(s)**	**Condition**	**Outcome**	**Molecule(s)**	**Result(s)**
Green et al. [264]	2014	Human	TDCA	Non-malignant oesophageal cells	Proliferation	CDX1 and CDX2	TDCA did not enrich proliferation markers
Bus et al. [267]	2014	Cell	DCA + GCA + TCDCA (mixture)	Non-malignant oesophageal cells	Proliferation	miR-143, miR-145 and miR-192	Mixture of BAs did not significantly change levels of miR-143, -145 and -192
BARRETT'S OESOPHAGUS							
Huo et al. [255]	2020	Human	DCA	BO	Proliferation	phospho-p38	DCA increased phospho-38
Wang et al. [227]	2018	Cell	DCA	BO	Proliferation	Notch 1	DCA suppressed Notch 1
Banerjee et al. [256]	2016	Human	UDCA	BO	Proliferation	Ki67	UDCA did not change Ki67 levels

Taddei et al. [254]	2014	Human	DCA	BO	Proliferation	VEGF	DCA induced proliferation via the induction of VEGF

Table 3.6.B. Bile-Acid Exposure and Proliferation Experiments in Oesophageal Adenocarcinoma

OESOPHAGEAL ADENOCARCINOMA							
Yamada et al. [257]	2014	Cell	DCA	OAC	Proliferation	CDX2	DCA activated CDX2
Chen et al. [258]	2020	Cell	DCA	OAC	Proliferation	KLF4 and OCT4	DCA promoted the expression of reprogramming factors KLF4 and OCT4
Kanai et al. [259]	2019	Cell	TCA	OAC	Proliferation	Calcein fluorescence ratio	TCA promoted cell proliferation in a dose-dependent manner
Liu et al. [203]	2018	Cell	TCA	OAC	Proliferation	S1PR2	TCA promoted cell proliferation. TCA also activated S1PR2
Matsuzaki et al. [260]	2013	Cell	CA and CDCA (separate experiments)	OAC	Proliferation	MiR-221, MiR-222 and CDX2	Both CA and CDCA exposure (separate experiments) increased levels of miR-221 and -222. CA and CDCA exposure degraded CDX2

Tables 3.6.A. and 3.6.B. Bile-Acid Exposure and Proliferation in Experiments on Non-Malignant Oesophageal Cells or Tissue, Barrett's Oesophagus and Oesophageal Adenocarcinoma

TDCA = taurodeoxycholic acid; CDX1 / 2 = Caudal Type Homeobox 1 / 2; DCA = deoxycholic acid; GCA = glycocholic acid; TCDCA = taurochenodeoxycholic acid; miR-143 / 145 / 192 = micro-RNA 143 / 145 / 192; BO = Barrett's Oesophagus; UDCA = ursodeoxycholic acid; VEGF = vascular endothelial growth factor; OAC = oesophageal adenocarcinoma; KLF4 = Krüppel-like Factor 4; OCT 4 = Octamer-binding transcription factor 4; TCA = taurocholic acid; S1PR2 = Sphingosine-1-phosphate receptor 2; CA = cholic acid; CDCA = chenodeoxycholic acid; miR-221 / 222 = micro-RNA 221 / 222.

Publications relating to Apoptosis and Apoptotic Resistance

Five studies investigated the effects of BAs on apoptosis [227], [255], [256], [260], [267] and 1 study investigated the effects of BAs on apoptotic resistance [251], see Table 3.7. Four studies were carried out on human cell-lines [227], [251], [260], [267] and 2 were based on human tissue [255], [256]. Two of these studies was carried out on non-malignant oesophageal cells [227], [267], 3 on BO [251], [255], [256] and 1 on OAC [260]. The effects of DCA on apoptosis were evaluated in 2 studies [227], [255] and the effects of DCA on apoptotic resistance were evaluated in 1 study [251]. One study each focused on the following BAs: UDCA; and CA and CDCA (separate experiments) [256], [260]. Another study incorporated a mixture of DCA plus GCA and TCDCA [267].

In non-malignant cell-lines, the mixture of BAs (DCA plus GCA and TCDCA) did not significantly change miR-143 levels or subsequent levels of apoptosis [267]. In a separate study on non-malignant oesophageal cells, DCA suppressed Notch 1 and hairy and enhancer of split 1 (Hes1): potentially BO-forming mechanisms [227]. DCA prevented apoptosis of BO tissue through increasing the expression of phospho-p38 [255]. DCA induced BO-related, apoptotic resistance through the action of NF-κB and Bcl-2 [251]. A study on UDCA did not note any change in cleaved caspase 3 (CC3), a marker of apoptosis, levels in patients with BO [256]. In OAC, CA and CDCA (separate experiments) increased levels of miR-221 and -222 [260]. By contrast, CA and CDCA exposure degraded the apoptosis-promoting CDX2 [260].

Table 3.7. Bile-Acid Exposure and Apoptosis and Apoptotic Resistance in Experiments on Non-Malignant Oesophageal Cells or Tissue, Barrett's Oesophagus and Oesophageal Adenocarcinoma

NON-MALIGNANT OESOPHAGEAL CELLS OR TISSUE							
Authors	**Year**	**Study Type**	**Bile Acid(s)**	**Condition**	**Outcome**	**Molecule(s)**	**Result(s)**
Wang et al. [227]	2018	Cell	DCA	Normal oesophageal cells	Apoptosis	Notch 1 and Hes 1	DCA suppressed Notch 1 and Hes 1
Bus et al. [267]	2014	Cell	DCA + GCA + TCDCA (mixture)	Normal oesophageal cells	Apoptosis	miR-143	Mixture of BAs did not significantly change miR-143 levels
BARRETT'S OESOPHAGUS							
Huo et al. [255]	2020	Human	DCA	BO	Apoptosis	phospho-p38	DCA increased phospho-p38, preventing apoptosis of BO
Feng et al. [251]	2017	Cell	DCA	BO	Apoptotic resistance	NF-κB and Bcl-2	DCA induced apoptotic resistance
Banerjee et al. [256]	2016	Human	UDCA	BO	Apoptosis	CC3	UDCA did not change CC3 levels

OESOPHAGEAL ADENOCARCINOMA							
Matsuzaki et al. [260]	2013	Cell	CA and CDCA (separate experiments)	OAC	Apoptosis	miR-221 and miR-222	Both CA and CDCA (separate experiments) increased levels of miR-221 and -222. CA and CDCA (separate experiments) degraded the apoptosis-promoting CDX2

Table 3.7. Bile-Acid Exposure and Apoptosis and Apoptotic Resistance in Experiments on Non-Malignant Oesophageal Cells or Tissue, Barrett's Oesophagus and Oesophageal Adenocarcinoma

DCA = deoxycholic acid; GCA = glycocholic acid; TCDCA = taurochenodeoxycholic acid; miR-143 = micro-RNA 143; BO = Barrett's Oesophagus; Notch 1 = neurogenic locus notch homolog protein 1; Hes 1 = hairy and enhancer of split-1; NF-κB = Nuclear factor kappa-light-chain-enhancer of activated B cells; UDCA = ursodeoxycholic acid; CA = cholic acid; CDCA = chenodeoxycholic acid; OAC = oesophageal adenocarcinoma; miR-221 / 222 = micro-RNA 221 / 222; CDX2 = caudal type homeobox 2

Publications relating to Angiogenesis

Two human studies focused on angiogenesis [254], [267], see Table 3.8. One examined effects on non-malignant oesophageal cells [267] and the other on BO [254]. The BO study examined the effects of DCA [254], while the non-malignant oesophageal cell study examined a mixture of DCA plus GCA and TCDCA [267].

DCA induced vascular endothelial growth factor (VEGF) expression [254], leading to angiogenesis in BO, while exposure to the BA mixture did not significantly change angiogenic miR-145 levels in non-malignant oesophageal cells [267].

Table 3.8. Bile-Acid Exposure and Angiogenesis in Non-Malignant Oesophageal Cells and Tissue and Barrett's Oesophagus Tissue							
Authors	**Year**	**Study Type**	**Bile Acid(s)**	**Condition**	**Outcome**	**Molecule(s)**	**Results**
Bus et al. [267]	2014	Human	DCA + GCA + TCDCA (mixture)	Non-malignant oesophageal cells	Angiogenesis	miR-145	BA exposure did not significantly change miR-145
Taddei et al. [254]	2014	Human	DCA	BO	Angiogenesis	VEGF	DCA induced VEGF expression

Table 3.8. Bile-Acid Exposure and Angiogenesis in Non-Malignant Oesophageal Cells or Tissue, Barrett's Oesophagus and Oesophageal Adenocarcinoma

DCA = deoxycholic acid; GCA = glycocholic acid; TCDCA = taurochenodeoxycholic acid; miR-145 = microRNA 145; BA = bile acid; BO = Barrett's Oesophagus; VEGF = vascular endothelial growth factor

5. Discussion

The goal of the present study was to examine the effects that BAs (individually or as a mixture), at neutral pH, might have on the risk or development of BO and OAC. Two systematic reviews on the topic (carried out in 2011 and 2012) indicated that BAs in media of various acidic pH conditions may play a role in the aetiology of these conditions [188], [191].

In the present systematic review, we examined 20 recent articles identified by a keyword search of 6 databases. Our key observations were as follows: DCA affected the biology of BO and OAC through a wide range of potentially co-operating mechanisms [226], [227], [251], [252], [253], [254], [255], [257], [258], [265], [266]; TDCA was associated with oxidative-stress generation and DNA damage in BO [209]; and UDCA prevented DCA-induced inflammation in non-malignant oesophageal cells and BO [226], [266]. Similar to the observations of prior systematic reviews, we also report a role for NF-κB, CDX1 and CDX2, KLF4 and MUC2 in inflammation, proliferation, apoptotic resistance or a combination of these outcomes.

The neutral-pH-related observations from the current systematic review largely align with those of McQuaid *et al*. [191]. The authors also noted an increase in oxidative stress, DNA damage, inflammation and proliferation upon BA exposure [191]; they did not, however, report OAC studies, angiogenic or apoptotic outcomes and did not report on the effects of UDCA. As such, the OAC-related, apoptotic, angiogenic and UDCA-related observations reviewed here merit further research. The concentrations used in the experiments from the current systematic review are overall lower (range: 1μM to 1,000μM, see Supplemental Table S2) than those reported in the studies from McQuaid *et al.* (5μM to 4,000μM) [191], making the observations in the current systematic review more physiologically relevant. There is also less heterogeneity among the studies in the current systematic review, strengthening the value of the results reported. The systematic review by Bus *et al.* mainly focused on BA exposure in conjunction with acidic environments and BA mixtures at a neutral pH: these mixtures proved to be toxic in all studies (non-malignant cells, BO and OAC) examined [188].

The Role of DCA in Tumour Biology

DCA and Oxidative Stress

In the observations from the present review, DCA was associated with oxidative-stress in BO [251], [252]. Reports in the literature have cited potential mechanisms underlying the DCA-related associations in non-oesophageal cancers and conditions [268], [269]. In colon epithelial cells, DCA induces mitochondrial oxidative stress through the activation of NF-κB [268]. DCA also induced oxidative stress in human colon adenocarcinoma cells, via the activation of NADPH oxidases [269].

DCA and DNA Damage

Observations from the present study indicate that DCA induced DNA damage in both non-malignant oesophageal cell-lines and in BO [226], [227], [253]. Additional studies examining the effect of DCA and DNA damage in the oesophagus exist in the literature [270], [271], [272]. In non-malignant oesophageal cells, DCA had a non-linear concentration response for DNA damage [270]; such a relationship suggests DNA damage to be dependent on the DCA concentration. A study on benign BO epithelial cells observed that DCA caused DNA damage [271]. One study focused on the effect of DCA on DNA damage and OAC development [272]: the DCA-related bile salt, sodium deoxycholate, was associated with increased DNA damage in both non-malignant and OAC cell-lines [272].

DCA and Inflammation

The studies included in the present systematic review report an increase in inflammation upon DCA exposure in non-malignant oesophageal cell-lines, BO cell-lines and tissue and OAC cell-lines [226], [227], [254], [257], [265], [266]. In the literature, a DNA-damage-related study noted that there was subsequent induction of inflammatory markers such as caspases, COX-2 promoter activity, NF-κB and Activator Protein 1 (AP-1) in colon-cancer cell-lines [273]. Therefore there may also be an interplay between oxidative-stress, DNA-damaging and inflammatory mechanisms in BO and OAC.

The Role of NF-κB in Cellular Inflammation

One of the key transcription factors involved in the induction of inflammation is NF-κB. Observations in the literature align with the observations of the present study [274], [275], [276], [277], [278]. At neutral pH, DCA induced NF-κB expression in oesophageal cell-lines [274], [275], [276], [277]. NF-κB is associated with a poor response to OAC chemotherapy [278], making it an attractive therapeutic target.

DCA and Cellular Proliferation

DCA affected cellular proliferation in 5 studies (3 on BO and 2 on OAC) in the present systematic review. DCA increased cellular proliferation in all 5 studies [227], [254], [255], [257], [258]. Evidence from the literature on other gut cancers also indicates increased proliferation in response to DCA exposure [279], [280], [281]. Ochsenkühn *et al.* noted that serum DCA promoted hyperproliferation of the colonic mucosa, a key precursor to the development of colon cancer [279]. Two studies stated the DCA doses at which proliferation of cancer of the colon occurred: 5μM and 20μM or 50μM, respectively [280], [281]. These concentrations are supraphysiological (see Supplemental Table S2), but consistent with the concentrations used in the 20 studies of the current systematic review.

The Role of CDX1 and CDX2 in Cellular Proliferation

CDX1 and CDX2 are transcription factors and play key roles in cellular proliferation [264]. Results from the studies in the current systematic review vary depending on the disease status of cell-lines and the individual BA involved [257], [260], [264]. There was no change in CDX1 and CDX2 levels (and subsequent proliferation) in non-malignant oesophageal cell-lines upon TDCA exposure [264]; perhaps longer exposure times (greater than the two 10-minute treatments per day for 3 days noted in the study in question) are needed for metaplastic features to occur. By contrast, DCA activated CDX2 in one OAC study [257]. In another OAC study, both CA and CDCA (separate experiments, concentration used 200μM CA or 10μM CDCA for 24 hours) led to the degradation of CDX2 by intracellular proteases [260]; CA and CDCA may be less toxic than DCA and may be tumour protective. The literature also cites a role for CDX1 and CDX2 in both the healthy and the diseased oesophagus [282], [283]. A BA

mixture (including CA, GCA and TCA) concentration-dependently increased *CDX1* promoter activity and CDX1 protein in non-malignant oesophageal epithelial cells [283]. A mixture of BAs (a total of 400μM containing GCA, TCA, GCDCA, TCDCA, GDCA and TDCA, exposed to cells 3 times a day for a week) induced *CDX2* mRNA and protein expression in oesophageal squamous cells from patients with BO, but not from GORD patients without BO [282]; such an observation highlights the importance of exposure time of BAs in the development of BO.

Proteins which partner with CDX2 (Notch proteins, Hes1 and atonal bHLH transcription factor 1 (ATOH1)) have also been described [284], [285], [286]. Notch proteins are thought to be tumour suppressive and Hes1 promotes Notch signalling. ATOH1 suppresses Hes1, and any subsequent Notch signalling. The tumour suppressive actions of Notch proteins are inactivated by DCA exposure (200μM for 8 hours) in non-malignant and OAC cell-lines, where there is a concomitant increase in CDX2 expression [285]. The tumour-promoting actions of ATOH are enhanced by DCA exposure (50μM to 200μM for 12 hours), again with a concomitant increase in CDX2 expression [286]. Exposure to DCA (100μM to 300μM for 8 hours), decreased Notch signalling; at the same time, *CDX2* mRNA and ATOH1 protein were increased 3-fold in OAC cells compared to untreated cells [284]. Since none of these studies employed proliferation assays, further research is needed to establish the role of these partner proteins in CDX2-related cancer proliferation. Experiments with lower DCA concentrations and longer DCA exposure times are warranted.

The Role of KLF4 in Cellular Proliferation

KLF4 is a transcription factor which regulates cell growth, proliferation, and differentiation [287]. When KLF4 is upregulated (by, for example, DCA), it can induce a phenotypic change in the oesophageal squamous epithelium to BO-like metaplasia [287]. Of note is that BAs increased the expression of KLF4 in oesophageal epithelial cells in a time-dependent manner [288]. In line with the above observations, human BO epithelium strongly expressed KLF4 [288]. Exposure to DCA (0-200 μM, 0-24 hours) induced gastric intestinal metaplasia (similar to oesophageal intestinal metaplasia) in gastric epithelial cells [211]; in the same

study, TGR5 was reported to play a role in this process through positively regulating KLF4 expression [211]. Investigating whether KLF4 is disproportionately expressed in males (as is TGR5) could provide vital information on how to treat male OAC patients.

The Role of MUC2 in Cellular Inflammation and Proliferation

MUC2 encodes for the most common gastrointestinal-tract gel-forming mucin 2 [289]. The gene can be activated by acid, or BAs, or a combination of these [289]. Mucin 2 has roles in both cellular proliferation and inflammation [289]. We note that MUC2 was upregulated by DCA in one study [227]. In an OAC cell-line study, *MUC2* mRNA expression was absent before exposure to DCA [289]; MUC2 expression subsequently increased in a concentration- (10 to 1000μM) and time-dependent (0-24 hour) manner with DCA exposure [289]; MUC2 may be playing a protective role upon DCA stimulation.

The Interplay of FXR, NF-κB, CDX2 and MUC2 in the Development of BO and OAC

Some studies in the literature identify an interaction between nuclear receptors (FXR), transcription factors (NF-κB, CDX2) and glycoproteins (MUC2) in the development of BO, OAC or gastric cancer [276], [289], [290], [291]. In a study by Hu *et al.*, CDX2 expression was increased, alongside MUC2 expression, upon DCA exposure (10μM to 1000μM concentrations) in non-malignant and OAC cell-lines [289]. An 18-hour incubation of OAC cells with DCA (at concentrations of 50μM to 300μM) increased the expression of both MUC2 and NF-κB [276]. Two studies on human gastric epithelial cells indicate that BA exposure (CDCA at 50 to 200μM for 24 hours or 400μM for an unreported exposure time) activated the FXR-NF-κB axis and increased the expression of CDX2 and MUC2 [290], [291]. The activation and expression of all of these molecules may initially be to protect from cancer development but overactivation by BAs may promote cancer development.

DCA and Apoptosis

Two studies examined the link between DCA exposure on apoptosis. Wang *et al*. reported that DCA suppressed Notch 1 and Hes 1 in normal oesophageal cells,

affecting the apoptosis of potentially malignant cells [227]. Huo *et al*. reported that DCA increased phospho-p38 in BO patients, preventing the apoptosis of BO [255]. Studies in the literature state that DCA can induce apoptosis, either promoting or preventing cancer [292], [293]. DCA (100μM to 500μM for up to 60 minutes) can induce apoptosis in a human colon-cancer cell-line [292]; such apoptosis disrupts the fine balance among proliferation, differentiation and apoptosis and is thought to be tumour-promoting [292]. By contrast, DCA (350μM for 36 hours) induced apoptosis in gastric-carcinoma cells through activation of an intrinsic mitochondria-dependent pathway [293].

DCA and Apoptotic Resistance

In line with an observation in the present study [251], one study in the literature noted that DCA induced apoptotic resistance in BO. In BO epithelial cells, DCA induced apoptotic resistance in cells with DNA damage; such resistance led to the increased likelihood of worsening BO and such worsening was induced by the activation of NF-κB [271].

DCA and Angiogenesis

In the present systematic review, DCA induced angiogenesis via the activation of VEGF in BO tissue [254]. A mixture of DCA, GCA and TCDCA in non-malignant human cell-lines did not significantly change miR-145 and subsequent angiogenesis [267]. Angiogenesis and related processes have been noted in cancer cells [294], [295]. Specifically, two studies reported that heparin-DCA conjugate can suppress angiogenesis and subsequent tumour growth [294], [295].

TDCA in BO and OAC Tumour Biology

TDCA, a conjugated form of DCA, was associated with oxidative-stress generation and DNA damage in BO cell-lines in the current study [209]. In non-malignant cell-lines, TDCA exposure did not affect CDX1 and CDX2 levels or subsequent proliferation [264]. Although we noted the generation of oxidative stress, there are a paucity of studies examining the relationship between TDCA exposure and oxidative stress. The data from the present study indicate that in BO, TDCA induces

DNA damage through the activation of the NADPH oxidase 5-S (NOX5-S) protein [209]. There is little published human or cellular information relating to TDCA-induced DNA damage in cancer. There is also little information relating TDCA exposure to CDX1 and CDX2 expression in non-malignant oesophageal cell-lines.

UDCA in Tumour Biology

UDCA and Inflammation

Evidence presented in the current review suggests that UDCA can attenuate DCA-induced inflammation, as indicated by reduced IL-6 and IL-8 expression [266] and increased GPX1 and catalase levels [226]. Literature supporting a link between UDCA and inflammation is the most comprehensive of any BAs studied. In macrophages, UDCA inhibited the pro-inflammatory responses induced by lipopolysaccharide [296]. The immunosuppressive action of UDCA has also been noted in dendritic cells [297]. Indeed, inhibiting the function of dendritic cells, through the BA-sensitive FXR, allows UDCA to suppress eosinophilic airway inflammation [241]. UDCA pre-treatment of OAC cells inhibited COX-2 upregulation, DCA-induced activation of NF-κB and AP-1 and also the translocation of NF-κB [275].

UDCA and Cell Proliferation

The lack of an effect of UDCA on proliferation markers is not consistent with observations from other tissue types. Martínez *et al*. and Serfaty *et al.* both noted the inhibition of cell proliferation by UDCA which prevented the development of colon cancer [298], [299]. A similar reduction in proliferation was observed in primary biliary cholangitis [300]; this study used the same proliferation marker, Ki67, as the report included in the present systematic review [256], [300]. Ki67 was also used to measure proliferation in a colorectal cancer model: UDCA inhibited tumour growth, as indicated by Ki67 levels, in a concentration-dependent manner [301].

UDCA and Apoptosis

The single study on UDCA and apoptosis in the present systematic review reported no change in the apoptotic marker, CC3 [256]. This observation contrasts with other

studies indicating that UDCA could enhance apoptosis [302], [303], [304]. This was reported in human melanoma [302], glioblastoma [303] and hepatocellular carcinoma [304]. UDCA either inhibited cancer-promoting apoptosis or promoted the programmed cell death of cancer cells [302], [303], [304], [305], [306], [307]. UDCA inhibited glioblastoma progression via inhibiting ER-stress-related apoptosis [303]. Two studies noted mitochondrial UDCA associations in human melanoma cells [302], [305]. Apoptosis is also activated in order to kill cancer cells [304], [306], [307]. UDCA induced apoptosis of hepatocellular carcinoma [304], [306]. One of these studies demonstrated that this programmed cell death was due to the activation, by UDCA, of the p53-caspase 8 pathway [306]. DCA-induced apoptosis can also be attenuated by the UDCA-related stimulation of Akt-dependent survival signalling [307].

UDCA as a Therapeutic Target

Given its tumour-protective characteristics, UDCA may be an appropriate therapeutic target; it could, for example, be administered orally to potentially change the profile of BAs in the stomach of BO and OAC patients. A human trial by Banerjee *et al*., however, demonstrated that high-dose UDCA supplementation for six months increased UDCA blood levels but did not modulate selected markers of oxidative stress, DNA damage, cell proliferation, and apoptosis in BO [256]. This lack of change may be due to the small sample size (29 patients), but a power calculation was not carried out [256].

The Role of BA Signalling in the Development of BO and OAC

Despite a clear presence of BA receptors in BO and OAC tissue, there is little research linking BA interaction with these receptors and subsequent metaplastic or oncogenic outcomes. The exception is research on S1PR2 [203], [245], [308], [309], [310], [311]. In the current systematic review, Liu *et al.* notes that TCA exposure activated S1PR2 and this in turn increased proliferation in OAC cells [203]. Cancer-related studies on BA interactions with S1PR2 focus on cholangiocarcinoma and hepatocellular carcinoma [245], [308], [309], [310], [311]; these studies demonstrate a role for TCA, TLCA, GCA and GDCA in particular. Liu *et al.*

demonstrated that TCA promoted cholangiocarcinoma-cell invasive growth through the activation of S1PR2 [309]; the same research group then showed that such S1PR2-mediated invasive growth involved the upregulation of COX-2 and PGE2 expression [308]. Amonyingcharoen *et al.* noted that TLCA induced cholangiocarcinoma cell growth [310]. Cholangiocarcinoma cells treated with GCA showed a 3.4-fold higher expression of S1PR2, compared to untreated cells [311]. Finally, GDCA upregulated the expression of S1PR2, promoting hepatocellular carcinoma [245]. Further research is needed to discern the potential role of the BA receptors FXR, TGR5, VDR, S1PR2 and PXR in the development of BO and OAC.

Role of BA concentration in the development of BO and OAC

The effects of BA exposure and the development of BO and OAC appears to be concentration-dependent [192]. The individual BA concentrations used in the studies in the current systematic review ranged from 1µM to 1000µM, while the concentrations reported by McQuaid *et al.*, ranged from 5µM to 1000µM [191]. We compared to concentrations used in the present systematic review with the following reference levels: mean serum concentrations (range: 0.025µM to 0.931µM) [192]; mean concentrations in gastric juice from patients with bile reflux (range: 0.03µM to 279.5µM) [189]; and typical median peak levels in the BO refluxate (range: <0.25µM to 39µM) [199]. These comparisons are summarized in Supplemental Table S2. In 20 experiments in the present systematic review, all concentrations used were higher than physiological levels. In 3 experiments, the concentration used was higher in 2/3 of physiological levels. In 5 experiments, some concentrations were higher than physiological levels. In 6 experiments, no comparison could be made (either because the concentration used was not expressed as a molarity or because of the use of BA mixtures). Further research is needed on the effects of BAs at concentrations matching the levels present in the healthy oesophagus, BO and OAC.

The Role of BA Exposure Durations and the Development of BO and OAC

The duration of BA exposure may also influence the development of BO and OAC [191]. The BA exposure times of the experiments in the current systematic review

ranged from 5 minutes to 12 months, whereas the exposure times in McQuaid *et al.* ranged from 3 minutes to 6 weeks [191]. The exposure times of included studies in the systematic review by Bus *et al.* ranged from 10 minutes to 7 days [188]. *In vivo* BO reflux episodes are approximately 3 minutes long and the mean frequency of reflux episodes over a 24-hour period is 154 [239]; similar exposure regimes have not been examined *in vitro*. There is likely a minimum exposure time of BAs in GORD patients before BO develops; experiments with longer exposure times are thus needed.

Limitations of the Present Study

The present study has limitations. Most (55%) of the studies included examined the potential effects of DCA on non-malignant oesophageal cells, BO and OAC [226], [227], [251], [252], [253], [254], [255], [257], [258], [265], [266]. More studies on non-DCA BAs need to be carried out in order to compare BA-specific results in a more balanced way. In contrast to DCA studies, relatively few studies (n=3) have examined the role of TDCA [209], [263], [264]. Only 1 of the reported studies was a randomized controlled trial [226]. There is thus a need for a human-subject study focused on BA profiles in OAC. Research on the interaction between BAs, BA receptors and BO and OAC development is also warranted. Despite the clear gender disparity associated with OAC in the literature [26], [210], and the sexual dimorphisms in cholesterol to BA conversion [312], no gender-related observations were made in the included studies of the present systematic review.

Potential for Intervention

The observations from the present systematic review lay the groundwork for further hypotheses, particularly relating to lifestyle intervention. Blood levels of BAs increase with increasing body mass index [313], [314]; as such, clinically managed weight loss may result in lower concentrations of toxic BAs [315]. UDCA supplementation lowers total cholesterol, the precursor for BA synthesis [316]. Similarly, beta glucan (found in oats) binds to BAs and cholesterol for excretion [317]. Plant sterols also inhibit intestinal absorption of cholesterol [318]. High-fat diets increase levels of DCA, raising the possibility that low-fat diets may reduce the

levels of these BAs or result in a more health-promoting profile [319]. Turmeric, containing the active compound curcumin, reduces levels of conjugated forms of DCA in those consuming a high-fat breakfast [320]. Furthermore, the pre-treating OAC cells with curcumin (50 μM) completely abolished the ability of DCA (300 μM) to activate NF-κB [321]. Since DCA consistently upregulates NF-κB [226], [251], [257], [268], [270], [271], [273], [276], [277], high polyunsaturated-fat diets or omega-3 supplementation may be possible therapeutic avenues [322]. Finally, diallyl disulfide, a compound found in garlic, attenuated DCA-induced inflammation and apoptotic resistance in BO [251]. Although, BA-targeting medications (BA sequestrants, Sucralfate, prokinetic agents and Baclofen) exist, lifestyle modifications are of greater appeal to cancer patients [323].

Conclusions

The current systematic review provides an update on previous evidence linking exposure to BAs (independently of acidity) and the development of BO and OAC; it also cites potential underlying mechanisms for the observed associations between individual BA exposure and BO and OAC biology. Our analysis highlights roles for DCA, TDCA, and UDCA. DCA exerted pro-oncogenic effects on non-malignant oesophageal tissue, BO and OAC through a wide range of cellular processes, including oxidative-stress generation, DNA damage, inflammation, proliferation, apoptosis, apoptotic resistance and angiogenesis [226], [227], [251], [252], [253], [254], [255], [257], [258], [265], [266]. TDCA was associated with the generation of oxidative stress and the damaging of DNA of cells in BO [209]. UDCA-exposure confers risk-reducing and protective effects on non-malignant oesophageal cells, BO and OAC [226], [256], [266] and may be a potential therapeutic target. We add to the range of scientific knowledge on the topic by collating previously under-reported results pertaining to OAC, apoptosis, angiogenesis and UDCA exposure [203], [226], [227], [251], [254], [256], [257], [258], [259], [260], [266], [267], [271]. We also demonstrate a role for NF-κB, CDX1, CDX2, KLF4 and MUC2 in oncogenesis (NF-κB, CDX1, CDX2 and KLF4) [226], [251], [257], [258], [260], [264] or tumour suppression (MUC2) [227]. Examining the associations of BAs at a neutral pH may help identify individuals at risk of progressing to BO or OAC and may present

prognostic avenues. The research on BAs and the development of BO and OAC could be strengthened by conducting randomized controlled trials, use of more physiologically relevant concentrations of BAs, lengthening of BA-exposure times, gathering of data on BA-BA-receptor interactions and collecting data on the male-female differences in BA effects. In summary, BAs play a role in the development of BO and OAC, independent of acidic environments, and could be targeted therapeutically through medication, bacterial supplementation, weight loss or dietary modification.

CHAPTER FOUR: DISCUSSION

1. Overall findings from the current work

The goal of the current work was to examine the potential role of Ca^{2+} signalling and BA-exposure in the development of BO and OAC. Understanding more about Ca^{2+} signalling in OAC may lead to a novel pharmacological treatment for the cancer, whereas understanding more about BA exposure in BO and OAC may also lead to potential lifestyle interventions.

Two studies were carried out as part of the current work: a transcriptomic analysis of the role of Ca^{2+} signalling proteins in OAC [324]; and a systematic review on the topic of the role of BAs in the development of BO and OAC [222]. The transcriptomic analysis identified six genes encoding Ca^{2+} signalling proteins that are associated with improved patient survival and three potential diagnostic markers for OAC [324]. The BA systematic review identified BAs, at a neutral pH, which have an association with the development of BO (DCA and TDCA) and OAC (DCA, TCA, CA and CDCA) [222]; UDCA was also identified as having a role in BO and OAC prevention [222].

2. Chapter 2: a transcriptomic analysis of Ca^{2+} signalling genes in OAC

My transcriptomic analysis of Ca^{2+} signalling genes in OAC identified six genes (*CACNA1D*, *CACNA2D4*, *JPH1*, *ACCN4*, *TRPM5* and *ATP2C2*) associated with improved patient survival and three genes (*GRIN2D*, *TRPC4* and *TRPM2*) with the top-ranking expression (across the two datasets examined) in OAC [324]; I also noted that the six survival-related genes were upregulated in advanced tumour grades and nodal metastatic stages [324]. In the literature, *CACAN1D* was associated with improved survival outcomes in colon adenocarcinoma [325] and *JPH1* in small-cell lung carcinoma [326]. The associations of the other genes with survival outcomes in the literature were either inconsistent with those of our study (*CACNA2D4*, *TRPM5* and *ATP2C2*) or unreported (*ACCN4*) [324].

I hypothesize that these six survival-associated genes may be either increasing tumor-cell death or inhibiting other cancer hallmarks, either directly or by indirect

mechanisms. In the literature, *JPH1* was targeted by miR-145 to favour tumour suppression [327], and *CACNA2D4* expression mitigated the adverse effects of chemotherapy in bromodomain containing 9 gastric cancer [328]. Ca^{2+}-dependent mechanisms have also been identified [329], [330], [331], [332], [333]. VGCCs, such as *CACNA1D* (encoding the $Ca_v1.3$ channel), have well documented roles in cell proliferation, migration, and apoptosis [329]. It is generally accepted that VGCCs regulate Ca^{2+} homeostasis in excitable cells following plasma-membrane depolarization [330]. Fourbon et al., however, showed, via gene silencing experiments in a colon-cancer cell line, that $Ca_v1.3$ regulated Ca^{2+} homeostasis and cell migration by a non-canonical function involving the plasma membrane Na^+/Ca^{2+} exchanger [330]; both canonical and non-canonical methods should be considered when investigating the impact of the channel on patient survival. TRPM5 (either the gene, *TRPM5*, the protein or both) regulated inflammatory responses (its presence reduced inflammation) in a Ca^{2+}-dependent manner [331], activated the Ca^{2+}-dependent production of mucous in goblet cells [333] and supported melanoma-to-lung metastasis through the Ca^{2+}-dependent production of Matrix Metalloproteinase 9 [74]; the latter result required activation by EA (greater than pH 5.9) [74]. For the most part, TRPM5 activation seems to be protective (this observation aligns with my Ca^{2+} signalling findings). It is not clear whether acidic environments (greater than pH 5.9) result in adverse TRPM5 outcomes in all cancers. Further research is required to discern the role of TRPM5 in OAC and in environments of varying pH. SPCA2 (the protein encoded by the *ATP2C2* gene) interacts with the SOCE channel, Orai1, and induces Ca^{2+} influx at the cell surface [332]; elevated SPCA2 expression could drive pro-survival and chemotherapy resistance in cancer cells [332]. There has been no research detailing Ca^{2+}-dependent, tumour-promoting mechanisms for *CACNA2D4*, *JPH1* or *ACCN4*.

The availability of pharmacological agonists or antagonists dictates the extent to which mechanistic experiments can be carried out on my genes of interest, especially on those (*CACNA1D*, *TRPM5* and *ATP2C2*) with an identified association with Ca^{2+} signalling and cancer formation. Pharmacological agonists or antagonists exist for the proteins arising from *CACNA1D* ($Ca_v1.3$) and *TRPM5* (Trpm5), but not

ATP2C2 (SPCA2). $Ca_v1.3$ antagonists include DHPs, Diltiazem, Phenylalkylamines and Pyrimidine-2,4,6-triones, whereas Bay K 8644 is an L-type Ca^{2+} channel agonist [334], [335], [336], [337], [338]. Agonists (Compound 39, PMID: 36402081; and Compound 64, a benzo[d]isothiazole derivative) and antagonists (flufenamic acid, quinine, zinc, triphenylphosphine oxide (TPPO) and extracellular protons) also exist for TRPM5 [74], [339], [340], [341], [342], [343], [344]. Despite the availability of agonists and antagonists for $Ca_v1.3$ and TRPM5, evidence surrounding their effectiveness in cancer and their potential side effects is limited.

Dihydropyridines (DHPs; such as nifedipine, amlodipine, felodipine, nimodipine and isradipine) block all L-Type VGCCS ($Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$ and $Ca_v1.4$) and sometimes other classes of VGCCs (amlodipine); such inhibition stops the voltage-dependent entry of Ca^{2+} into the cell. DHPs are potent vasodilators [345]. They dilate arterial smooth muscle in resistance vessels, reduce peripheral vascular resistance, lower arterial blood pressure and antagonize vasospasms in coronary or peripheral arteries [334]. Inhibition of L-type VGCCs by DHPs is a well-established treatment of hypertension and cardiac ischemia [334]. There have been no studies on DHP use among OAC patients. Nifedipine use decreased distal oesophagus contraction amplitude in patients with nutcracker (hypercontractile) oesophagus [346]. The limiting side-effects of DHPs are predominantly hypotension, peripheral oedema and constipation [334].

Not all DHPs are Ca^{2+} channel antagonists. Bay K 8644, a DHP, is an L-type Ca^{2+} channel agonist [335]. It has been shown to have a positive inotropic effect (it increases the force of muscular contractions) [347]. It is used in laboratory experiments to induce a rise in intracellular Ca^{2+} [348], but has not been overly studied in disease treatment. Side-effects of Bay K 8644 administration include altered heart rate, motor impairment, and behavioural changes [349], [350], [351].

Diltiazem is a benzothiazepine subclass of Ca^{2+} channel blocker [336]. It also targets L-Type VGCCs [352]. Diltiazem is a widely prescribed Ca^{2+} antagonist drug for cardiac arrhythmia, hypertension, and angina pectoris [353]. Because of its smooth-

muscle-relaxing effect, its role in treating diffuse oesophageal spasm has been investigated [336], [352]. A randomized controlled trial (n=125), carried out by Forootan et al., demonstrated that diltiazem relieved clinical symptoms in patients with distal oesophageal spasm [352]. Another smaller study (10 patients vs. 5 controls) noted minimal effects from diltiazem use and oesophageal spasm alleviation [336]. There are no studies investigating the potential role of diltiazem in cancer.

Phenylalkylamines (including verapamil, gallopamil and devapamil) are blockers of L-Type VGCCs [337], [354]. They are used in the treatment of ischaemic heart disease, arrythmia and hypertension [345]. Verapamil use has been shown to reverse chemoresistance in leukaemia, colon cancer, hepatocellular carcinoma, and breast cancer cell lines [355], [356], [357], [358]. When combined with docetaxel or vincristine, verapamil use resulted in the cell death of chemo-resistant lung cancer cells [359].

Of key interest is that novel $Ca_v1.3$-selective blockers (specifically: pyrimidine-2,4,6-triones) are being researched [334], [338]. This research is particularly pertinent given that $Ca_v1.3$ channels have been implicated in the pathogenesis of Parkinson's disease [338].

Agonists (Compound 39, PMID: 36402081; and Compound 64, a benzo[d]isothiazole derivative) and antagonists (flufenamic acid, quinine, zinc, triphenylphosphine oxide (TPPO) and extracellular protons) also exist for TRPM5 [74], [339], [340], [341], [342], [343], [344]. Given that Compound 39 and Compound 64 have only been recently researched [339], [340], their potential role in disease treatment has not yet been studied. Flufenamic acid is an analgesic drug used to relieve pain associated with rheumatoid diseases [360]. Its potential role in cancer treatment has not been studied. Quinine inhibits the taste transduction function of TRPM5 [342]. It has been shown to have an antiproliferative and pro-apoptotic effect in both cervical and lung cancer cell-lines [361]. Physiological concentrations of zinc inhibit TRPM5 function [343]. Research has linked a zinc-TRPM5 interaction to both

gastric-cancer and OSCC development [362], [363], [364]. A significant portion (69%) of gastric cancer patients exhibit serum zinc deficiency [364], affecting the cadmium:zinc ratio and resulting in DNA damage [363]. Similarly, zinc deficiency contributed to the progression of OSCC [362]. Orai1 is highly expressed in OSCC and results in hyperactive intracellular Ca^{2+} oscillations and cell proliferation; zinc treatment inhibits this process. TPPO is a potent and selective inhibitor of TRPM5 [74], [344]. Its study on cancer has been limited. TRPM5 current is completely blocked at pH 5.9 [365]. Maeda et al. demonstrated that TRPM5 activity at a pH level greater than 5.9 results in lung metastasis in a mouse B16-BL6 melanoma cell-line [74], whereas my study demonstrated an association with improved patient survival in OAC [324]. Such observations illustrate the diversity of characteristics of distinct forms of cancer and also highlights the important of experimentation at multiple pH levels. I do not have data on the pH level of the OAC tumors examined in the OCCAMS dataset (Chapter 2).

There does not seem to be known agonists or antagonists of SPCA2. The availability of agonists and antagonists allows for more comprehensive laboratory experiments to be carried out.

Because sustained and intermittent acid exposure is what differentiates OAC from many cancers [366], the potential role of EA in OAC is again brought into focus. Acidic environments can induce changes in $[Ca^{2+}]_c$ [24]. During acid reflux, the pH of the distal oesophagus can drop from about pH 6.5 to approximately pH 2 [62]. It has been shown that EA acts as a signal for the transformation of squamous to glandular cells in BO [367].

Acidic pH has been reported to regulate Ca^{2+}-permeable ion channels in a direct and indirect way, via specific H^+ binding sites or via competition with Ca^{2+} ions for binding sites [368]. A limited number of studies have been carried out on the effects of EA exposure and Ca^{2+} signalling in OAC [174], [175], [176], [177]. Relatively little is known about whether the six survival-associated genes identified in Chapter 2 sense acid and subsequently affect $[Ca^{2+}]_c$ levels [324]. *TRPM5* has the most well-

documented association and *ACCN4* has a tentative, indirect association (via interaction of the ASIC4 protein with ASIC1a, a known pH-sensitive cation channel) [324]. *CACNA1D, CACA2D4, JPH1* and *ATP2C2* do not have any known pH-sensing function [324]. Decreases in extracellular pH either quickly block TRPM5-induced current (it is completely blocked at pH 5.9) or slowly enhance current inactivation; the former is reversible while the latter is irreversible [365]. Both a complete block or a slow enhancement of current inactivation have the potential to affect Ca^{2+} signalling and this may lead to oncogenesis. More research on the potential effect of acidic environments on the function of my shortlisted genes.

2.1. Potential roles of the shortlisted Ca^{2+} signalling genes in OAC metastasis

Cancer metastasis is defined as the spread of cancer from the site of origin to another area of the body [138]. Epidemiological research focusing exclusively on OAC metastasis is scant. A large proportion (37%) of all OACs are diagnosed at Stage IV of the TNM system [369]. The five-year relative survival for metastatic OC (grouped as OSCC and OAC, 61% OAC in one study [370]) is less than 5% [370], [371]. This compares to the five-year survival of 41% and 23%, respectively, for localized (exclusively in the oesophagus) and regional (spread to nearby lymph nodes or tissue) oesophageal cancer (grouped) [370]. The worst outcomes (including mortality rate) for OAC were associated with Stage IV tumours [369]. In the current study, all six OAC survival-associated genes (*CACNA1D*, *CACNA2D4*, *JPH1*, *ACCN4*, *TRPM5*, *ATP2C2*) were upregulated, at least to a certain extent, in various OAC nodal metastatic stages compared to normal tissue [324]. This observation is pertinent as it demonstrates the potential for pharmacological intervention (perhaps to enhance the function or upregulate the levels of these Ca^{2+} signalling proteins) to halt not only OAC formation but also tumour progression. It is currently unclear why these six genes are associated with improved patient survival, but they are also highly expressed in metastatic stages. It is possible that the genes are limiting the deleterious effects of metastasis. Investigating whether metastatic tumours or cells with high expression of our survival-associated genes respond

better to treatment than tumours with low expression may provide valuable insights into the prognostic value of therapeutics targeting the genes in question.

Chapter 2 details the associations between Ca^{2+} signalling and metastasis until 31st December 2021; some pertinent research has been carried out since. Two studies [372], [373] focused on *CACNA1D* expression in prostate cancer. In a publication by O'Reilly et al., *CACNA1D* expression was significantly increased in metastatic tumours, compared to primary tumours, and metastatic tumours had increased *CACNA1D* expression compared with the adjacent normal samples [372]. In a publication by McKerr et al., significant *CACNA1D* overexpression was found in primary and metastatic prostate tumours compared to adjacent benign prostate tissue [373]. Furthermore, *CACNA1D* overexpression was detected across all Gleason grades in primary and metastatic prostate cancer, compared to adjacent benign tissue [373]. Such observations are consistent with those of my transcriptomic analysis [324] and highlight the significance of *CACNA1D* expression in the progression of prostate cancer and the potential value as a therapeutic target. Post-2021, metastasis-related research has also been carried out on *JPH1* expression [374]. *JPH1* mRNA was present at increased quantities in lung metastatic tissues as compared to primary tumours of the breast. There has been no further OAC metastasis research relating to *CACNA2D4*, *ACCN4*, *TRPM5* and *ATP2C2* function since 2021.

2.2. Perspectives on Ca^{2+} signalling, BO and OAC

Taken together, expression of my six survival-associated genes could span across all disease stages and could be potential prognostic markers [324]. Given the ubiquitous expression of *GRIN2D* in OAC and OSCC, *TRPC4* and *TRPM2* may be more selective, diagnostic markers for OAC [324]. Ca^{2+} signalling is a relatively understudied therapeutic target and targeting a Ca^{2+} signalling pathway would alter cellular function in a different way to classic chemotherapies and may have fewer or more tolerable side-effects. Given the strong association of OAC with EA [31], [375], any potential chemotherapy would either need to be tested under acidic conditions or be used in conjunction with acid neutralisation or suppression. The

availability of agonists and antagonist for some of the Ca^{2+} signalling proteins would aid in designing comprehensive laboratory experiments. Figure 4.1. illustrates the key research findings of the present Ca^{2+} signalling, transcriptomic analysis accompanied by the next research steps and the clinical relevance of the research.

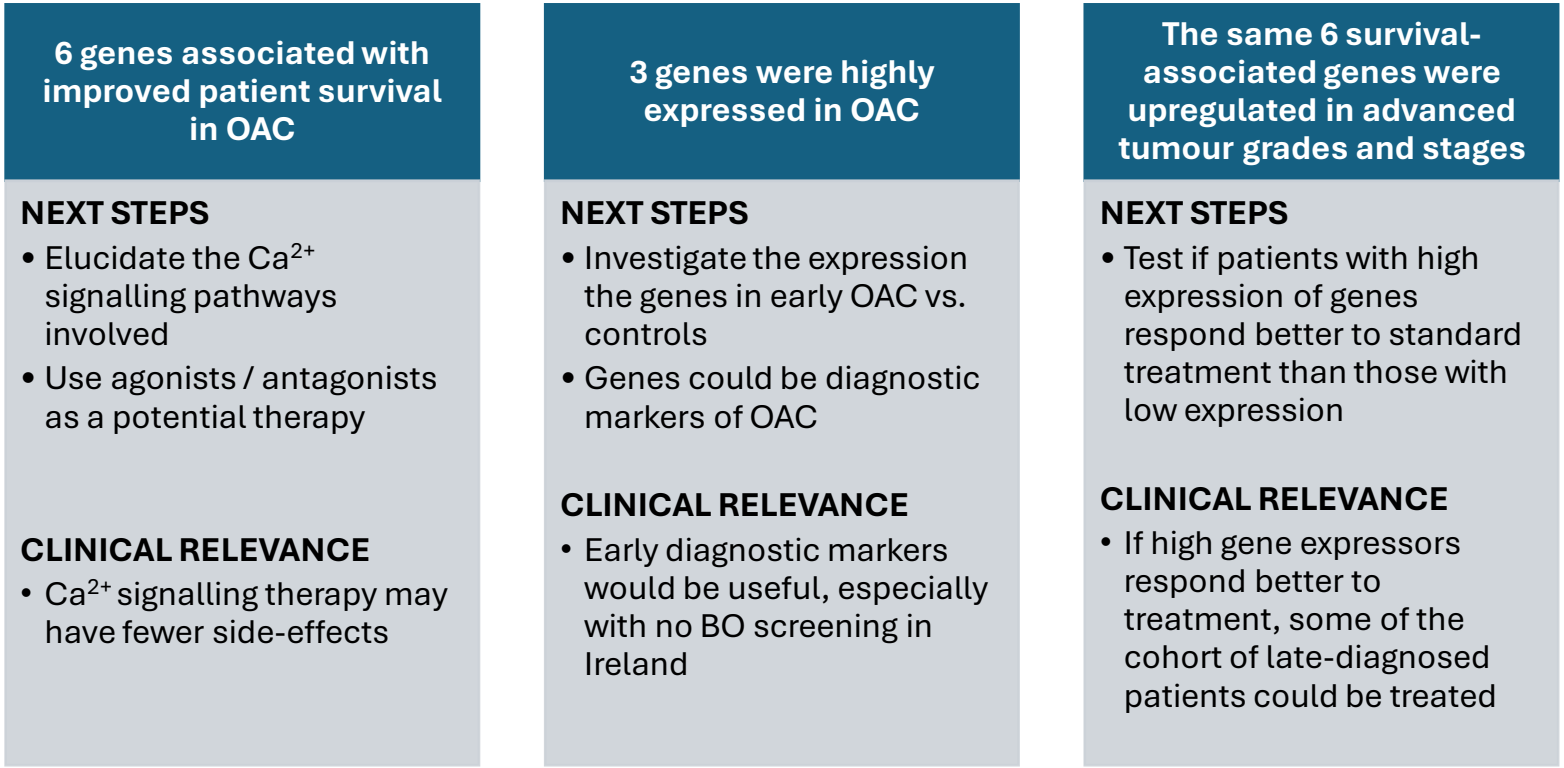

Figure 4.1. Key research findings of the present Ca^{2+} signalling, transcriptomic analysis accompanied by the next research steps and the clinical relevance of the research

OAC = oesophageal adenocarcinoma; BO = Barrett's oesophagus; 6 genes = *CACNA1D, CACNA2D4, JPH1, ACCN4, TRPM5, ATP2C2*; 3 genes = *GRIN2D, TRPC4, TRPM2*

3. Chapter 3: the role of BAs in BO and OAC

The incidence of BO and OAC continues to rise despite the prescribing of acid-suppressing and acid-neutralising medication [228], [229]. This brings into question the possibility that BAs, independently of the effects of acidity, might be contributing to the development of OAC. I carried out a systematic review on the role of neutral-pH, BA exposure in development of BO and OAC [222]. This systematic review identified BAs having an association with BO (DCA and TDCA) and OAC (DCA, TCA, CA and CDCA) development; UDCA was also identified as having a role in BO and OAC prevention. Additionally, I noted that BA-induced BO and OAC was associated with potentially co-operating mechanisms such as oxidative stress, DNA damage, inflammation, proliferation, apoptosis, apoptotic resistance and angiogenesis. Similar to the observations of prior systematic reviews, I also reported a role for NF-κB, CDX1 and CDX2, KLF4 and MUC2 in inflammation, proliferation, apoptotic resistance or a combination of these outcomes. MUC2 also had a role in tumour suppression.

3.1. Added value of the present systematic review

My study has added to the range of scientific knowledge on the topic by collating previously under-reported results pertaining to OAC, apoptosis, angiogenesis and UDCA exposure [203], [226], [251], [254], [255], [257], [258], [259], [266]. In OAC, DCA induced inflammatory markers [257] and both DCA and TCA activated several proliferation factors [203], [257], [258], [259]. DCA either induced apoptotic resistance or decreased beneficial apoptosis in BO [251], [255]. DCA induced VEGF expression, leading to angiogenesis, in BO [254]. UDCA exposure prevented DNA damage in BO [226]; attenuated DCA-induced inflammation in non-malignant oesophageal cells [266]; and upregulated anti-inflammatory enzymes in BO [226]. Expanding our knowledge on these topics brings us closer to identifying markers of BO to OAC progression, whilst the prophylactic effects of UDCA may result in a viable lifestyle modification (UDCA supplementation) for at-risk individuals.

3.2. Oncogenic roles of BA signalling receptors

The biological mechanisms underpinning BA-induced BO and OAC in the current systematic review could be mediated through the action of BA-signalling receptors such as FXR, TGR5 and VDR [192], [202], [206]. FXR expression appears to be tumour-promoting, TGR5 to have pleiotropic effects (depending on BA involved) and the VDR appears to be tumour protective [192], [200], [206], [210], [236], [247], [301], [376], [377], [378], [379]. Overexpression of FXR is linked with increased proliferation of cancer cells [376], whilst inhibition of FXR suppressed tumour cell viability, induced apoptosis and reduced tumour formation [247]. The hydrophobic CDCA is the most efficacious ligand of FXR [192]; I noted that CDCA exposure was associated with increased proliferation and reduced apoptosis of OAC cells in a study of the present systematic review (Chapter 3). *TGR5* is more highly expressed in HGD and OAC than in BO or LGD [200]. Patients with high expression of TGR5 exhibited significantly worse overall survival compared to the patients with low expression [210]. DCA, the most commonly examined BA in my collated studies, has the third highest potency of all BAs at TGR5 [377]. Most of TGR5-related, BA-exposure research has focused on TDCA and UDCA exposure [210], [236], [301], demonstrating pleiotropic effects of the receptor. Overexpression of TGR5 significantly enhanced the effects of TDCA in an OAC cell-line: TDCA bound to TGR5 and induced NOX5-S expression, which in turn increased cell proliferation, ROS production and DNA damage [236]. UDCA (concentration: 400μM to 700 μM) has been shown to inhibit colorectal cancer cell proliferation through the TGR5-mediated suppression of YAP signalling [301]. Examining the link between UDCA, TGR5 and tumour suppression and TDCA, TGR5 and tumour promotion warrants further study. The VDR has been significantly correlated with TGR5 expression [210]. *VDR* mRNA expression was higher in BO, compared with squamous epithelium [378], and higher expression of the VDR protein was detected in both BO and OAC tissue, compared to squamous epithelium [202]. VDR has an affinity for dehydro-LCA, LCA, CDCA and DCA, respectively [206]. In analysis adjusted for confounders, higher VDR expression was associated with an improved overall survival and disease-specific survival, when comparing the highest with the lowest tertile of expression [379]. The rs1989969T/rs2238135G haplotype of *VDR* gene was associated with an approximately two-fold reduced risk of reflux

oesophagitis, BO and OAC [378]. This *VDR* haplotype could be useful in identifying individuals who benefit most from vitamin D chemoprevention.

3.3. Potential role of BA receptors in the gender disparity of BO and OAC incidence

Alterations in the expression of BA signalling receptors (specifically, TGR5 and VDR) may explain the disproportionate incidence of OAC and BO in males [210]. OAC is nine times more common in males than females [26] and BO is two to four times more common in males than in females [380]. Furthermore, female OAC patients have longer survival than male patients [381]. TGR5 expression has been shown to be 1.9 times higher in male OAC patients compared to female OAC patients [210] and VDR expression was consistently higher in precancerous lesions and OAC in males than in females [202]. BA exposure relaxes the lower oesophageal sphincter through interaction with TGR5 [240]; such an interaction would increase the amount of refluxate reaching the oesophagus at a given time. High TGR5 expression was positively associated with KLF4 expression in tissue with intestinal metaplasia (a BO characteristic) [211]. Investigating whether KLF4 is disproportionately expressed in males could provide vital information on how to treat male OAC patients; this is especially pertinent given that the present systematic review reported KLF4 to be upregulated by DCA in OAC. The conundrum presented in the literature is how TGR5 and VDR expression could be disproportionately expressed in males, could be highly correlated with each other yet have opposing survival outcomes with high expression. This observation may be explained by different mutations within the respective receptors [378], [382], their BA binding affinities [192], [383], allosteric binding [382] or the differences in the functional effects of nuclear (VDR) and membrane-bound receptors (TGR5) [383]. It would appear that TGR5 function is over-riding VDR function in male OAC cases, contributing to worsened survival, but this hypothesis requires further study. Gender-related differences in BA signalling are nevertheless a key contender in explaining the differences in incidence of BO and OAC in males versus females [210]. This follows research (from three separate studies, [83], [384], [385]) on the potential role of circulating sex hormones (up to twelve hormones examined per study) and sex-

hormone receptors (oestrogen receptor alpha, oestrogen receptor beta and androgen receptor) in the male-female disparity in the incidence of OAC. For the most part, no association was noted for the steroid hormones examined [384], [385]. Xie et al. noted that higher endogenous levels of sex-hormone binding globulin and lower levels of follicle-stimulating hormone may increase patient survival in OAC [384]. The sex hormone receptor study by McMenamin et al. revealed only a moderate association with oestrogen receptor beta expression and OAC [83]; there was no association with the other hormone receptors examined. Oestrogen, progesterone, testosterone, vitamin D and BAs are all steroid hormones [386] and they all could be competitively binding to a number of types of BA receptors at a given time. Indeed, Sato et al. have documented that progesterone, testosterone and 17-beta oestradiol (the most potent form of oestrogen) are TGR5 agonists [387].

3.4. Potential links between BA exposure, Ca^{2+} signalling and cancer formation

Chapter 2 of the current work details the known associations between low pH environments and Ca^{2+} signalling (mediated by Ca^{2+} Toolkit proteins) and cancer formation [324]. It is likely that BA exposure also induces changes in Ca^{2+} signalling in the cell and this may result in cancer formation. The literature surrounding this topic is sparse at best, with no BA-dependent, Ca^{2+} signalling and cancer study documented to date. Ca^{2+} signalling regulates the formation of bile and orchestrates secretion of bile from both hepatocytes and cholangiocytes (bile duct epithelial cells) [388]. Impaired Ca^{2+} signalling is common in cholestasis [388]. Several Ca^{2+}-signalling proteins have been implicated these processes: Orai1, ryanodine receptors (RYRs) and IP_3Rs [388], [389], [390], [391], [392], [393], [394], [395], [396], [397], [398], [399], [400], [401]. Study on the relationship between BA exposure and Ca^{2+} signalling has been carried out in the pancreas, the liver, immune cells and smooth muscle cells [388], [389], [390], [391], [392], [393], [394], [395], [396], [397], [398], [399], [400], [401]. In the pancreas, intracellular Ca^{2+} elevation regulates digestive enzyme secretion in acinar cells and fluid and ion secretion in ductal cells [394]. BAs act on acinar cells, activating Ca^{2+} toxicity and

resulting in acute pancreatitis [394]. BAs induce necrosis in pancreatic stellate cells dependent on Ca^{2+} entry and sodium-driven bile uptake [395]. Increased and unbalanced ROS production caused by sustained Ca^{2+} elevation further contributes to cell dysfunction, leading to mitochondrial damage and cell death [396]. There appears to be a clear role of SOCE in BA-induced Ca^{2+} toxicity [392], [394], [397], [398], [399]. SOCE channels are the principal Ca^{2+} influx channels that contribute to Ca^{2+} overload in pancreatic acinar cells [392], [394], [398], [399]. BA activation of Orai1 damages pancreatic ductal secretion in acute pancreatitis [394]. Orai1 inhibition prevents progression of acute pancreatitis to chronic pancreatitis and also prevents extracellular Ca^{2+} influx, protecting cells from injury [399]. There also appears to be a clear role of RYR Ca^{2+}-release channels in BA-induced Ca^{2+} signalling in the pancreas [400], [401]. BAs (particularly, TCA) activate RYRs in pancreatic acinar cells via a direct allosteric mechanism [400]. The severity of biliary pancreatitis may be ameliorated by the clinical use of RYR inhibitors [401]. In the liver, intracellular Ca^{2+} regulates hepatocyte secretion of bile via IP_3R-mediated Ca^{2+} signalling [390]. Loss of IP_3Rs may be a final common pathway for cholestasis [390]. At physiological concentrations, TUDCA potently modulates $[Ca^{2+}]_c$ signals in hepatocytes by mobilizing microsomal IP_3-sensitive Ca^{2+} stores by an IP_3-independent mechanism [391]. This in turn initiates Ca^{2+} oscillations and induces the influx of extracellular Ca^{2+} [391]. In immune cells, BAs are vital for tuning innate immune responses against infections [392]. In CD4+ T cells, BAs disrupt intracellular Ca^{2+} homeostasis via the inhibition of mitochondria Ca^{2+} uptake and the elevation of $[Ca^{2+}]_c$ [392]. This leads to STIM1 and ORAI1 decoupling and impaired SOCE [392]. Impaired SOCE signalling halts T-cell activation [392]. Finally, in smooth muscle cells, natural BA and synthetic analogues increase large conductance Ca^{2+}-activated K+ (BKCa) channel activity [393]; such activation results in an efflux of K+ ions, the hyperpolarization of the plasma membrane, the closing of VGCCs and the halting of Ca^{2+} entry to the cell [402]. Such activation results in the relaxation of smooth muscle cells [402].

3.5. Summary of associations between BAs, BO and OAC

Overall, research into the role of BA exposure, independent of acidic pH, is needed given the persistence of BO and OAC in the wake of acid-suppressing and acid-neutralising medication [228], [229]. My systematic review revealed under-reported associations linking BA exposure to OAC, apoptosis, apoptotic resistance and angiogenesis, as well as UDCA exposure to tumour suppression [203], [226], [251], [254], [255], [257], [258], [259], [266]. I have highlighted a role for several cancer biology mechanisms and propose that these mechanisms are underpinned by BA signalling receptors [192], [202], [206]. I also support the suggestion that disproportionate expression of BA signalling receptors may explain the disproportionate incidence of BO and OAC in males [210]. Finally, despite details of how BA exposure affects Ca^{2+} in normal physiological functioning [388], [389], [390], [391], [392], [393], [394], [395], [396], [397], [398], [399], [400], [401], I note a lack of studies linking BA exposure to Ca^{2+} signalling and subsequent cancer formation. Figure 4.2. illustrates the key research findings of the present BA and OAC systematic review, accompanied by the next research steps and the clinical relevance of the research.

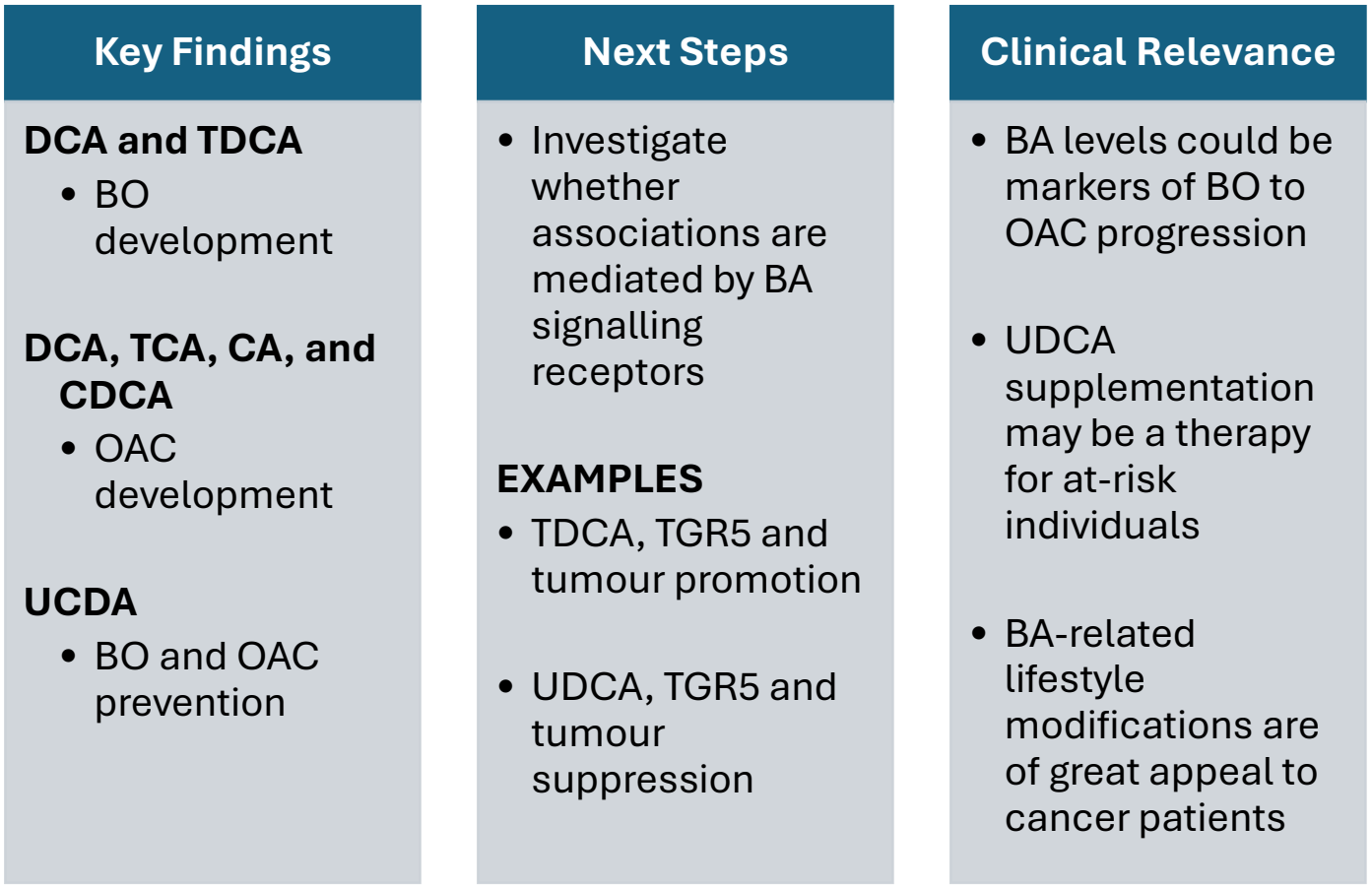

Figure 4.2. Key research findings of the present bile acid (BA) and OAC systematic review, accompanied by the next research steps and the clinical relevance of the research

DCA = deoxycholic acid; TDCA = taurodeoxycholic acid; BO = Barrett's oesophagus; TCA = taurocholic acid; CA = cholic acid; CDCA = chenodeoxycholic acid; OAC = oesophageal adenocarcinoma; UDCA = ursodeoxycholic acid; BA = bile acid; TGR5 = Takeda-G-protein-receptor-5

4. Future directions

The research carried out as part of this Research Master's lays the groundwork for further investigations. Research into the Ca^{2+} signalling related mechanisms underlying the positive patient-survival-associated genes may identify therapeutic targets and research into BA exposure may unveil promising lifestyle modification. The potential interactions between the following partners in OAC may yield promising results: *CACNA1D* with Ca_v subunits, the *RYR1* and the G protein-coupled oestrogen receptor; *JPH1* and miR-145; *ACCN4* and *GPR65*; *TRPM5* and *NCX*s; *ATP2C2* and *ORAI1*; and *TRPC4* and *GPR68* [324]. The role of the TME (particularly the acidic component) should also be considered in the design of such experiments [324]. Given that TRPM5 has the strongest known acid-sensing function (with currents being completely blocked at pH 5.9) [365], and is also activated by conjugated BAs [403], particular focus should be given to this this protein. One such area of investigation is the potential of TRPM5 to induce the Ca^{2+}-dependent production of mucous in goblet cells (found in BO and to a lesser extent in OAC) [333]. The main mucous-producing gene, *MUC5AC*, is mutated (loss of heterozygosity) in 61% of OAC patients (the fourth-highest ranking mutation in OAC) [47]. Interestingly, *TRPM5* and *MUC5AC* share the same chromosomal location: 11p15.5 [404], [405]. It is not known whether *TRPM5* mutations (LOH or otherwise) are common in BO or OAC. What we do know is that the gene is exclusively expressed from the paternal allele [406], again alluding to the gender disparity in OAC. It is not clear whether acidic environments above pH 5.9 activate TRPM5 in a tumour-promoting or tumour-suppressive way. Depending on the influence of acidic environments, a possible treatment for BO and OAC could be acid-neutralisation, alteration of the BA profile, followed by activation (by Compound 39 or Compound 64) of TRPM5. Among the potential interventions to modify harmful BA profiles include weight loss, UDCA supplementation, increasing beta glucan intake, increasing plant sterol intake, following low-fat diets, increasing turmeric intake, omega-3 supplementation and increasing garlic intake. Figure 4.3. summarizes the key findings of the present research, accompanied by proposed next steps and the clinical relevance of such research.

Key Findings	Next Steps	Clinical Relevance
6 Ca^{2+} signalling genes associated with improved patient survival	Focus the potential of TRPM5 to Ca^{2+} dependently produce mucous in goblet cells of the oesophagus If the TRPM5 gene is mutated in patients, gene therapy may be an option	Producing mucous would protect the oesophagus against acid and BA exposure and allow oesophageal tissue to heal Producing mucous would not have the side-effects of classic chemotherapies
Certain BAs associated with OAC development Certain BAs associated with OAC prevention	**LIFESTYLE INTERVENTIONS** • Maintaining a healthy weight • UDCA supplementation • Oats (beta glucan) • Plant sterols • Low-fat diets • Turmeric (curcumin) • Omega-3 supplementation • Garlic (diallyl sulfide)	Lifestyle interventions are of greater appeal to cancer patients

Figure 4.3. Summary of the key findings of the present research, accompanied by proposed next steps and the clinical relevance of such research

BAs = bile acids; TRPM5 = Transient Receptor Potential Cation Channel Subfamily M Member 5; UDCA = ursodeoxycholic acid

CHAPTER FIVE: OVERALL CONCLUSIONS

In conclusion, both Ca^{2+} signalling and BA exposure play a role in the development of BO and OAC; investigating the role in these conditions may present novel diagnostic or therapeutic avenues. The results presented in the current work lay the groundwork for highly informed laboratory research hypotheses. The prevalence, predicted growth and patient survival rate of OAC, along with the histological differences to OSCC, make the cancer a worthy research pursuit, especially given the fact the cancer in question has a precursor condition: BO [1], [4], [6], [232], [407]. New therapies are urgently needed [114]. Ca^{2+} signalling is a relatively understudied therapeutic. Targeting a Ca^{2+} signalling pathway would alter cellular function in a different way to classic chemotherapies and may have fewer or more tolerable side-effects. I noted that six Ca^{2+} signalling genes were associated with improved OAC patient survival [324]. I noted that these six genes were also associated with OAC metastasis [324], highlighting the potential of these genes to halt not only OAC development, but also OAC progression. Since GORD is a major risk factor for BO and OAC development [33], [408], my studies focused on refluxed contents of the stomach: digestive acid and BAs [33]. The functions of my six shortlisted survival-associated genes in acidic environments in BO and OAC should be investigated further. Whilst acid exposure (alone or in conjunction with BAs) has been linked to BO and OAC development, its role in the aetiology of disease has not explained all cases [199], [228], [229]. Elucidating the effects of BAs, independent of pH, was therefore of key importance. I noted several BAs associated with the prevention or development of BO and OAC and propose that the role of BA signalling receptors be examined further. My BA study has added to the range of scientific knowledge on the topic by collating previously under-reported results pertaining to OAC, apoptosis, angiogenesis and UDCA exposure [203], [226], [251], [254], [255], [257], [258], [259], [266]. BAs may be viable diagnostic markers or could be targeted therapeutically through medication, bacterial supplementation, weight loss or dietary modification. In both OAC and BO, the male:female disparity warrants further study. Eventually, one may be able to link acid or BA exposure to altered Ca^{2+} signalling (via the observed Ca^{2+} signalling proteins) and BO and OAC development; such observations would be a valuable step to establishing a viable

new treatment options. A possible treatment regimen might involve acid neutralisation or suppression, alteration of the BA profile, targeting Ca^{2+} signalling genes or a combination of these. The possibility of a reduced side-effect therapy and lifestyle interventions are of great appeal to cancer patients [323].

CHAPTER SIX: REFERENCES (CHAPTERS ONE, THREE AND FOUR)

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CHAPTER SEVEN: APPENDICES

Appendices from Chapter Two

Supplemental Table S1. List of 275 Ca^{2+}-Toolkit Genes Analysed in both the UALCAN and OCCAMS Datasets	
Gene:	**Protein Name:**
ACCN1	Acid-sensing Ion Channel 2
ACCN2	Acid-sensing Ion Channel 1
ACCN3	Acid-sensing Ion Channel 3
ACCN4	Acid-sensing Ion Channel 4
ACTN1	Actinin α 1
ACTN2	Actinin α 2
ACTN3	Actinin α 3
ACTN4	Actinin α 4
AHCYL1	Adenosylhomocysteinase-Like 1
AKAP5	A-Kinase Anchoring Protein 5
AKAP6	A-Kinase Anchoring Protein 6
AKAP9	A-Kinase Anchoring Protein 9
ANXA1	Annexin A1
ANXA10	Annexin A10
ANXA11	Annexin A11
ANXA13	Annexin A13
ANXA2	Annexin A2
ANXA3	Annexin A3
ANXA4	Annexin A4
ANXA5	Annexin A5
ANXA6	Annexin A6

ANXA7	Annexin A7
ANXA8	Annexin A8
ANXA9	Annexin A9
ASPH	Aspartate β-Hydroxylase
ATF4	Activating Transcription Factor 4
ATP2A1	Sarco-endoplasmic-reticulum Ca^{2+} ATPase 1
ATP2A2	Sarco-endoplasmic-reticulum Ca^{2+} ATPase 2
ATP2A3	Sarco-endoplasmic-reticulum Ca^{2+} ATPase 3
ATP2B1	Plasma Membrane Ca^{2+} ATPase 1
ATP2B4	Plasma Membrane Ca^{2+} ATPase 4
ATP2C1	Secretory Pathway Ca^{2+} ATPase 1
ATP2C2	Secretory Pathway Ca^{2+} ATPase 2
ATP6V0A1	ATPase H^{+} Transporting V0 Subunit A1
ATP6V0A2	ATPase H^{+} Transporting V0 Subunit A2
ATP6V0A4	ATPase H^{+} Transporting V0 Subunit A4
ATP6V0B	ATPase H^{+} Transporting V0 Subunit B
ATP6V0C	ATPase H^{+} Transporting V0 Subunit C
C22ORF32	Essential MCU Regulator
CA1	Carbonic Anhydrase 1
CA10	Carbonic Anhydrase 10
CA11	Carbonic Anhydrase 11
CA12	Carbonic Anhydrase 12
CA13	Carbonic Anhydrase 13
CA14	Carbonic Anhydrase 14
CA2	Carbonic Anhydrase 2

CA3	Carbonic Anhydrase 3
CA4	Carbonic Anhydrase 4
CA6	Carbonic Anhydrase 6
CA7	Carbonic Anhydrase 7
CA8	Carbonic Anhydrase 8
CA9	Carbonic Anhydrase 9
CACNA1A	Voltage-Gated Ca^{2+} Channel, P/Q Type, A 1A Subunit.
CACNA1B	Voltage-Gated Ca^{2+} Channel, N-Type, A 1B Subunit.
CACNA1C	Voltage-Gated Ca^{2+} Channel, L-Type, A 1C Subunit.
CACNA1D	Voltage-Gated Ca^{2+} Channel, L-Type, A 1D Subunit.
CACNA1H	Voltage-Gated Ca^{2+} Channel, T-Type, A 1H Subunit.
CACNA2D1	Voltage-Gated Ca^{2+} Channel Auxiliary Subunit A 2 Δ 1
CACNA2D2	Voltage-Gated Ca^{2+} Channel Auxiliary Subunit A 2 Δ 2
CACNA2D3	Voltage-Gated Ca^{2+} Channel Auxiliary Subunit A 2 Δ 3
CACNA2D4	Voltage-Gated Ca^{2+} Channel Auxiliary Subunit A 2 Δ 4
CACNB1	Voltage-Gated Ca^{2+} Channel, L-Type, β 1 Subunit
CACNB2	Voltage-Gated Ca^{2+} Channel, L-Type, β 2 Subunit
CACNB3	Voltage-Gated Ca^{2+} Channel, L-Type, β 3 Subunit
CACNB4	Voltage-Gated Ca^{2+} Channel, L-Type, β 4 Subunit
CACNG4	Voltage-Gated Ca^{2+} Channel Auxiliary Subunit Gamma 4
CALB1	Calbindin 1
CALB2	Calbindin 2
CALM1	Calmodulin 1
CALM2	Calmodulin 2
CALM3	Calmodulin 3

CALR	Calreticulin
CAMK1	Calmodulin-dependent Protein Kinase 1
CAMK1D	Calmodulin-dependent Protein Kinase 1D
CAMK2A	Calmodulin-dependent Protein Kinase 2A
CAMK2B	Calmodulin-dependent Protein Kinase 2B
CAMK2D	Calmodulin-dependent Protein Kinase 2D
CAMK4	Calmodulin-dependent Protein Kinase 4
CANX	Calnexin
CASQ2	Calsequestrin 1
CBARA1	Mitochondrial Ca^{2+} Uptake 1
CCDC109A	Mitochondrial Ca^{2+} Uniporter
CCDC90A	Mitochondrial Ca^{2+} Uniporter Regulator 1
CD38	Cluster of Differentiation 38
CHRNA1	Nicotinic Cholinergic Receptor A1
CHRNA10	Nicotinic Cholinergic Receptor A10
CHRNA3	Nicotinic Cholinergic Receptor A3
CHRNA5	Nicotinic Cholinergic Receptor A5
CHRNA7	Nicotinic Cholinergic Receptor A7
CHRNA9	Nicotinic Cholinergic Receptor A9
CNGA1	Cyclic Nucleotide Gated Channel Subunit α 1
CNGB1	Cyclic Nucleotide Gated Channel Subunit β 1
CREB1	CAMP Responsive Element Binding Protein 1
CREB3	CAMP Responsive Element Binding Protein 3
CREB3L1	CAMP Responsive Element Binding Protein 3-Like 1
CREB3L2	CAMP Responsive Element Binding Protein 3-Like 2

CREB3L3	CAMP Responsive Element Binding Protein 3-Like 3
CREB3L4	CAMP Responsive Element Binding Protein 3-Like 4
CREB5	CAMP Responsive Element Binding Protein 5
EFHA1	Mitochondrial Ca^{2+} Uptake 2
EFHA2	Mitochondrial Ca^{2+} Uptake Family Member 3
FKBP1A	12 kDa FK506-Binding Protein
FKBP1B	12.6 kDa FK506-Binding Protein
GNA11	G Protein Subunit α 11
GNA14	G Protein Subunit α 14
GNAQ	G Protein Subunit α Q
GNB1	G Protein Subunit β 1
GNB2	G Protein Subunit β 2
GNB3	G Protein Subunit β 3
GNB4	G Protein Subunit β 4
GNB5	G Protein Subunit β 5
GNG10	G Protein Subunit Gamma 10
GNG11	G Protein Subunit γ 11
GNG12	G Protein Subunit γ 12
GNG2	G Protein Subunit γ 2
GNG3	G Protein Subunit γ 3
GNG4	G Protein Subunit γ 4
GNG5	G Protein Subunit γ 5
GNG7	G Protein Subunit γ 7
GOPC	Golgi-Associated PDZ and Coiled-coil Motif Containing
GPR132	G Protein-Coupled Receptor 132

GPR4	G Protein-Coupled Receptor 4
GPR65	G Protein-Coupled Receptor 65
GPR68	G Protein-Coupled Receptor 68
GRIN1	N-Methyl-D-Aspartate Receptor 1
GRIN2A	N-Methyl-D-Aspartate Receptor Subunit 2A
GRIN2D	N-Methyl-D-Aspartate Receptor Subunit 2D
HOMER1	Homer Scaffold Protein 1
HOMER2	Homer Scaffold Protein 2
HOMER3	Homer Scaffold Protein 3
HSP90AA1	Heat Shock 90kDa Protein 1, α
HSP90AB1	Heat Shock 90kDa Protein 1, β
HSPA13	Heat Shock 70kDa Protein A13
HSPA14	Heat Shock 70kDa Protein A14
HSPA1A	Heat Shock 70kDa Protein 1A
HSPA1B	Heat Shock 70kDa Protein 1B
HSPA1L	Heat Shock 70kDa Protein 1L
HSPA2	Heat Shock 70kDa Protein A2
HSPA4	Heat Shock 70kDa Protein A4
HSPA5	Heat Shock 70kDa Protein A5
HSPA6	Heat Shock 70kDa Protein A6
HSPA7	Heat Shock 70kDa Protein A7
HSPA8	Heat Shock 70kDa Protein A8
HSPA9	Heat Shock 70kDa Protein A9
HVCN1	Hydrogen Voltage Gated Channel 1
ITPR1	Inositol 1,4,5-Trisphosphate Receptor Type 1

ITPR2	Inositol 1,4,5-Trisphosphate Receptor Type 2
ITPR3	Inositol 1,4,5-Trisphosphate Receptor Type 3
JPH1	Junctophilin 1
JPH2	Junctophilin 2
JPH3	Junctophilin 3
JPH4	Junctophilin 4
KCNIP2	Potassium Voltage-Gated Channel Interacting Protein 2
KCNIP3	Potassium Voltage-Gated Channel Interacting Protein 3
KCNIP4	Potassium Voltage-Gated Channel Interacting Protein 4
LETM1	Mitochondrial Proton/Ca^{2+} Exchanger Protein
MCOLN1	Mucolipin 1
MCOLN2	Mucolipin 2
MRVI1	Murine Retrovirus Integration Site 1 Homolog
NCS1	Neuronal Ca^{2+} Sensor 1
NFATC1	Nuclear Factor of Activated T Cells 1
NFATC2	Nuclear Factor of Activated T Cells 2
NFATC3	Nuclear Factor of Activated T Cells 3
NFATC4	Nuclear Factor of Activated T Cells 4
ORAI1	Ca^{2+}-Release Activated Ca^{2+} Modulator 1
ORAI2	Ca^{2+}-Release Activated Ca^{2+} Modulator 2
ORAI3	Ca^{2+}-Release Activated Ca^{2+} Modulator 3
P2RX7	Purinergic Receptor P2X, Ligand Gated Ion Channel, 7
PICK1	Protein Interacting with C Kinase 1
PKD1	Polycystin 1
PKD2	Polycystin 2

PLCB1	Phospholipase C β 1
PLCB2	Phospholipase C β 2
PLCB3	Phospholipase C β 3
PLCD1	Phospholipase C δ 1
PLCD3	Phospholipase C δ 3
PLCD4	Phospholipase C δ 4
PLCE1	Phospholipase C ε 1
PLCG1	Phospholipase C γ 1
PLN	Phospholamban
PPP1CA	Protein Phosphatase 1 Catalytic Subunit α
PPP2CA	Protein Phosphatase 2 Catalytic Subunit α
PPP3CA	Protein Phosphatase 3 Catalytic Subunit α
PPP3CB	Protein Phosphatase 3 Catalytic Subunit β
PPP3CC	Protein Phosphatase 3 Catalytic Subunit γ
PRKCG	Protein Kinase C γ
PRKCI	Protein Kinase C ι
PRKCZ	Protein Kinase C ζ
PVALB	Parvalbumin
RGS1	Regulator of G Protein Signalling 1
RGS16	Regulator of G Protein Signalling 16
RGS2	Regulator of G Protein Signalling 2
RGS4	Regulator of G Protein Signalling 4
RYR1	Ryanodine Receptor 1
RYR2	Ryanodine Receptor 2
RYR3	Ryanodine Receptor 3

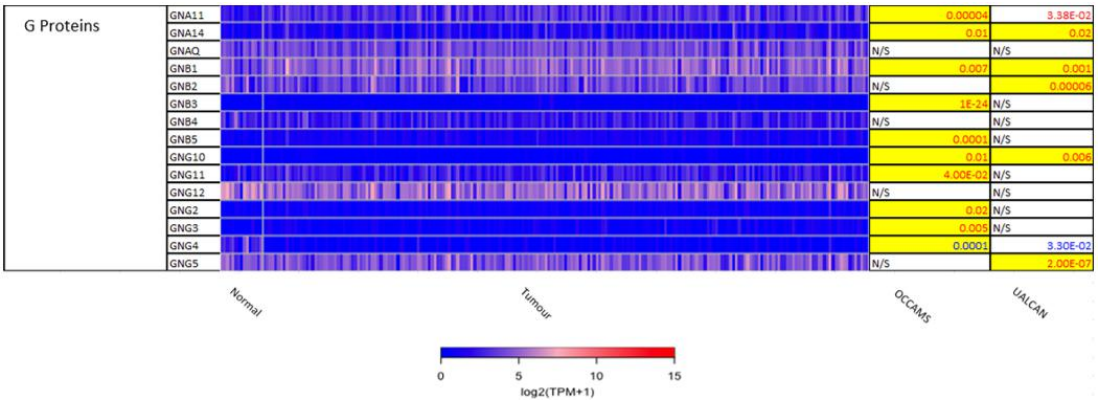
S100A1	S100 Ca^{2+} Binding Protein A1
S100A10	S100 Ca^{2+} Binding Protein A10
S100A11	S100 Ca^{2+} Binding Protein A11
S100A12	S100 Ca^{2+} Binding Protein A12
S100A13	S100 Ca^{2+} Binding Protein A13
S100A14	S100 Ca^{2+} Binding Protein A14
S100A16	S100 Ca^{2+} Binding Protein A16
S100A2	S100 Ca^{2+} Binding Protein A2
S100A3	S100 Ca^{2+} Binding Protein A3
S100A4	S100 Ca^{2+} Binding Protein A4
S100A5	S100 Ca^{2+} Binding Protein A5
S100A6	S100 Ca^{2+} Binding Protein A6
S100A7	S100 Ca^{2+} Binding Protein A7
S100A8	S100 Ca^{2+} Binding Protein A8
S100A9	S100 Ca^{2+} Binding Protein A9
S100B	S100 Ca^{2+} Binding Protein B
S100P	S100 Ca^{2+} Binding Protein P
SEPN1	Selenoprotein N1
SLC24A1	Sodium-Potassium-Ca^{2+} Exchanger 1
SLC24A3	Sodium-Potassium-Ca^{2+} Exchanger 3
SLC24A5	Sodium-Potassium-Ca^{2+} Exchanger 5
SLC24A6	Na(+)/K(+)/Ca(2+)-Exchange Protein 6
SLC25A4	ADP/ATP translocase 1
SLC25A5	ADP/ATP translocase 2
SLC26A1	Solute Carrier Family 26 Member 1

SLC26A10	Solute Carrier Family 26 Member 10
SLC26A2	Solute Carrier Family 26 Member 2
SLC26A3	Solute Carrier Family 26 Member 3
SLC26A4	Solute Carrier Family 26 Member 4
SLC26A5	Solute Carrier Family 26 Member 5
SLC26A6	Solute Carrier Family 26 Member 6
SLC26A7	Solute Carrier Family 26 Member 7
SLC26A8	Solute Carrier Family 26 Member 8
SLC26A9	Solute Carrier Family 26 Member 9
SLC4A1	Solute Carrier Family 4 Member 1 (Diego Blood Group)
SLC4A10	Solute Carrier Family 4 Member 10
SLC4A11	Solute Carrier Family 4 Member 11
SLC4A2	Solute Carrier Family 4 Member 2
SLC4A3	Solute Carrier Family 4 Member 3
SLC4A4	Solute Carrier Family 4 Member 4
SLC4A5	Solute Carrier Family 4 Member 5
SLC4A7	Solute Carrier Family 4 Member 7
SLC4A8	Solute Carrier Family 4 Member 8
SLC4A9	Solute Carrier Family 4 Member 9
SLC8A1	Sodium-Ca^{2+} Exchanger 1
SLC8A2	Sodium-Ca^{2+} Exchanger 2
SLC9A1	Sodium/Hydrogen Exchanger 1
SLC9A2	Sodium/Hydrogen Exchanger 2
SLC9A3	Sodium/Hydrogen Exchanger 3
SLC9A4	Sodium/Hydrogen Exchanger 4

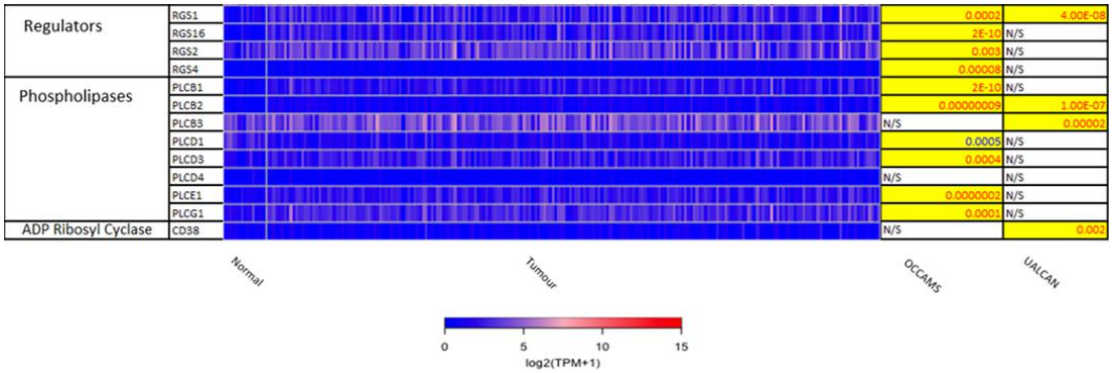
SLC9A5	Sodium/Hydrogen Exchanger 5
SLC9A6	Sodium/Hydrogen Exchanger 6
SLC9A7	Sodium/Hydrogen Exchanger 7
SLC9A8	Sodium/Hydrogen Exchanger 8
SLC9A9	Sodium/Hydrogen Exchanger 9
SRI	Sorcin
STIM1	Stromal Interaction Molecule 1
STIM2	Stromal Interaction Molecule 2
STOML1	Stomatin-Like 1
STOML2	Stomatin-Like 2
TPCN1	Two Pore Segment Channel 1
TPCN2	Two Pore Segment Channel 2
TRPA1	Transient Receptor Potential Cation Channel Subfamily A Member 1
TRPC1	Transient Receptor Potential Ion Channel Subfamily C Member 1
TRPC4	Transient Receptor Potential Ion Channel Subfamily C Member 4
TRPC6	Transient Receptor Potential Ion Channel Subfamily C Member 6
TRPM2	Transient Receptor Potential Ion Channel Subfamily M Member 2
TRPM4	Transient Receptor Potential Ion Channel Subfamily M Member 4
TRPM5	Transient Receptor Potential Cation Channel Subfamily M Member 5
TRPM6	Transient Receptor Potential Ion Channel Subfamily M Member 6
TRPM7	Transient Receptor Potential Ion Channel Subfamily M Member 7
TRPV1	Transient Receptor Potential Ion Channel Subfamily V Member 1
TRPV2	Transient Receptor Potential Ion Channel Subfamily V Member 2
TRPV3	Transient Receptor Potential Ion Channel Subfamily V Member 3
TRPV4	Transient Receptor Potential Cation Channel Subfamily V Member 4

TRPV5	Transient Receptor Potential Cation Channel Subfamily V Member 5
TRPV6	Transient Receptor Potential Ion Channel Subfamily V Member 6
VDAC1	Voltage Dependent Anion Channel 1
VDAC2	Voltage Dependent Anion Channel 2
VDAC3	Voltage Dependent Anion Channel 3

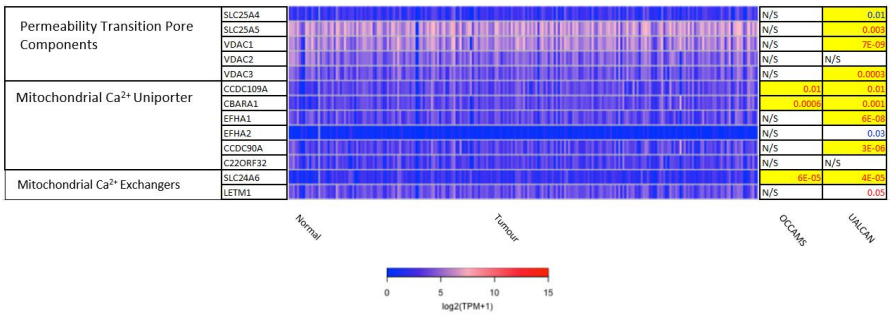
A)



B)

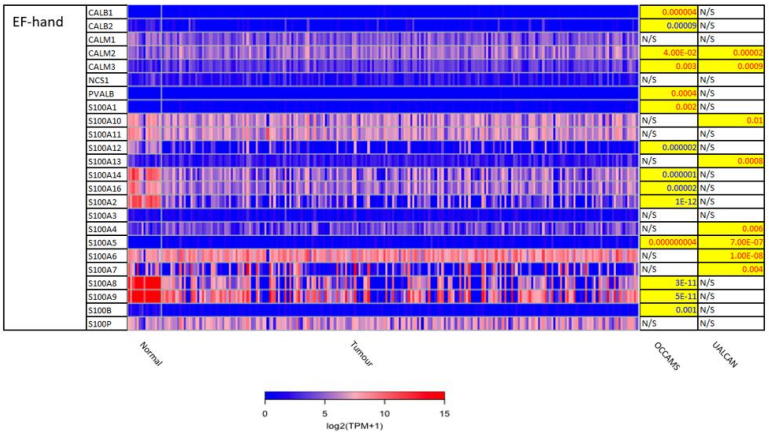


Supplemental Figure S1. Expression of Ca^{2+}-Signal Transducers in OAC. Heatmap depicting the expression-levels of genes encoding proteins involved in Ca^{2+} signal transduction: G Proteins (*Panel A*) and Regulators, Phospholipases and ADP Ribosyl Cyclase (*Panel B*). *Please see Figure 2.2 for details.* Expression data on GNG7 was not available from the OCCAMS dataset and was downregulated in the UALCAN data (*P* = 3.42e-2; not significant after adjustment for MOT).

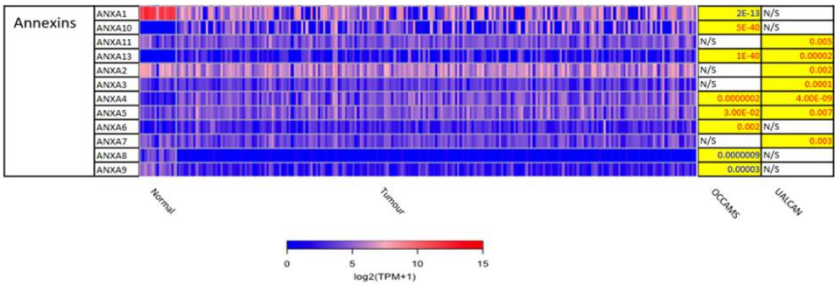


Supplemental Figure S2. Expression of Mitochondria-Associated, Ca^{2+}-Toolkit Genes in OAC. *Please see Figure 2.2 for details*.

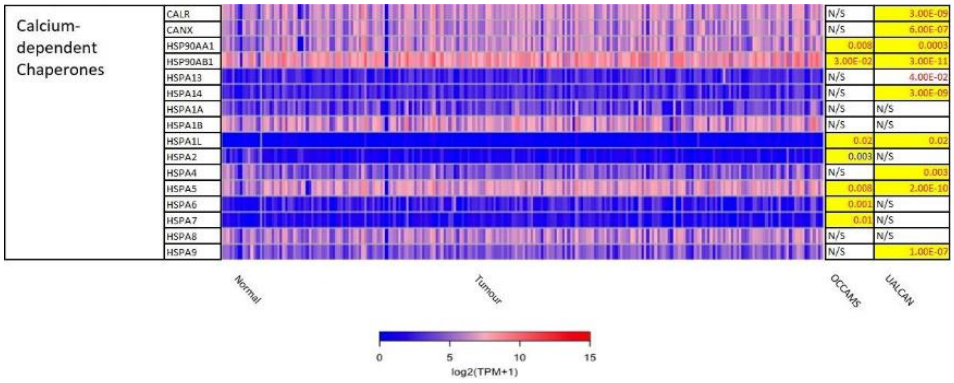
A)



B)

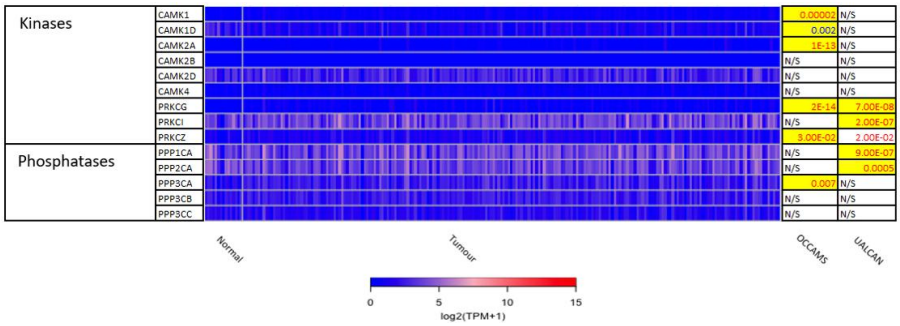


Supplemental Figure S3. Expression of Cytosolic Ca^{2+}-sensors and -buffers in OAC. *Please see Figure 2.2 for details.* SRI was not available in the OCCAMS data but was upregulated in the UALCAN data (*P* = 1e-8).

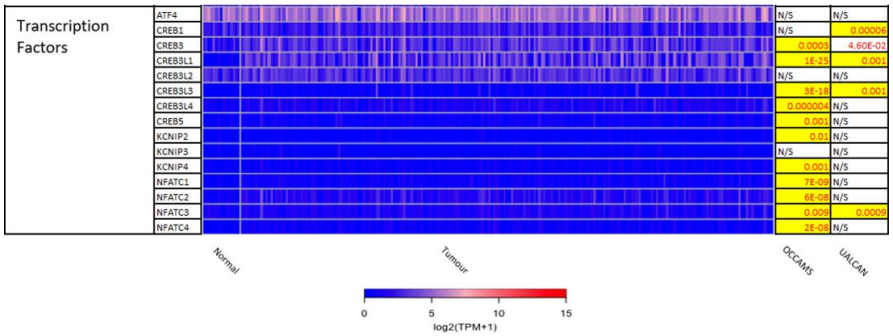


Supplemental Figure S4. Expression of Ca^{2+}-dependent Chaperones in OAC. *For details, please see Figure 2.2*

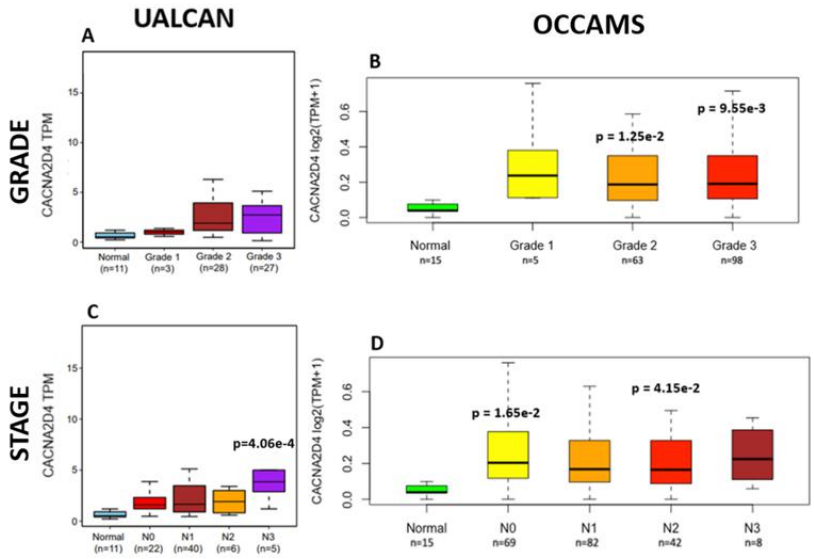
A)



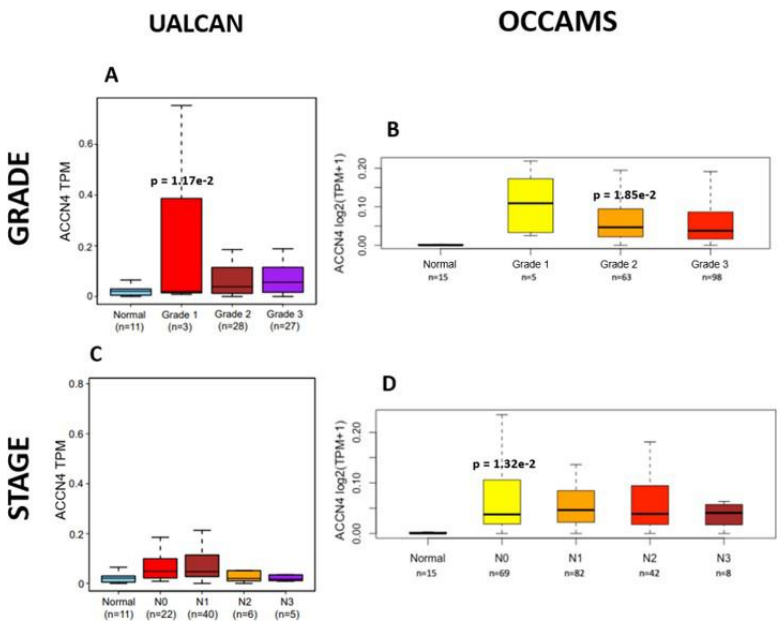
B)



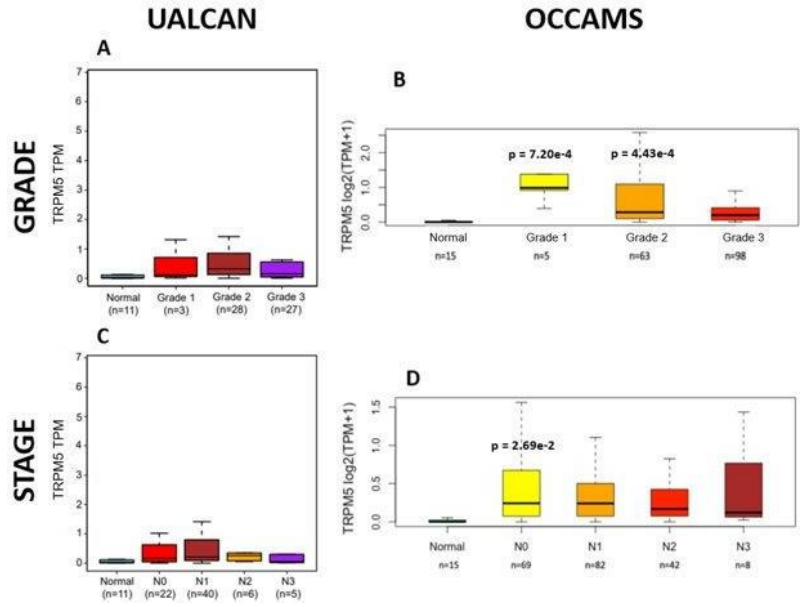
Supplemental Figure S5. Expression of Ca^{2+}-dependent effectors in OAC. *See Figure 2.2 for details.*



Supplemental Figure S6. OAC-Tumour, Grade and Nodal-Metastatic-Stage Boxplots for CACNA2D4. Details as described in *Figure 2.7*



Supplemental Figure S7. OAC-Tumour, Grade and Nodal-Metastatic-Stage Boxplots for ACCN4. Details as described in *Figure 2.7*



Supplemental Figure S8. OAC-Tumour, Grade and Nodal-Metastatic-Stage Boxplots for TRPM5. Details as described in *Figure 2.7*

CHAPTER SEVEN: APPENDICES

Appendices from Chapter Three

SUPPLEMENTAL TABLE S1. LIST OF KEYWORDS USED IN DATABASE SEARCHES
CONCEPT ONE: OESOPHAGEAL ADENOCARCINOMA
Oesophageal adenocarcinoma
Esophageal adenocarcinoma
OAC
EAC
Oesophageal cancer
Esophageal cancer
OC
EC
Oesophageal tumour
Esophageal tumour
Oesophageal tumor
Esophageal tumor
Oesophageal neoplasm
Esophageal neoplasm
Gastro oesophageal junction
Gastro esophageal junction
S1 OR S2 OR S3 OR S4 OR S5 OR S6 OR S7 OR S8 OR S9 OR S10 OR S11 OR S12 OR S13 OR S14 OR S15 OR S16
CONCEPT TWO: BARRETT'S OESOPHAGUS
Barrett's Oesophagus
Barrett's Esophagus
Barrett's
Barrett's Metaplasia
BO

BE
BM
S18 OR S19 OR S20 OR S21 OR S22 OR S23 OR S24
CONCEPT THREE: BILE ACID / ACID EXPOSURE
Bile acid
BA
bile acid exposure
Bile salt
BS
Bile salt exposure
Primary bile acid
Primary bile salt
Secondary bile acid
Secondary bile salt
Conjugated bile acid
Conjugated bile salt
Unconjugated bile acid
Unconjugated bile salt
Bile acid derivative
BA derivative
Bile salt derivative
BS derivative
Cholic acid
CA
Chenodeoxycholic acid
CDCA
Deoxycholic acid
DCA

Lithocholic acid
LCA
Glycocholic acid
GCA
Glycochenodeoxycholic acid
GCDCA
Glycodeoxycholic acid
GDCA
Glycolithocholic acid
GLCA
Taurocholic acid
TCA
Taurochenodeoxycholic acid
TCDCA
Taurodeoxycholic acid
TDCA
Taurolithocholic acid
TLCA
Ursodeoxycholic acid
UDCA
Gastro oesophageal reflux disease
Gastro esophageal reflux disease
GORD
GERD
Bile acid reflux
BA reflux
Bile salt reflux
BS reflux

Reflux
Refluxate
Acid
Acid reflux
Hydrochloric acid
Hydrochloric acid reflux
HCl
HCl reflux
Stomach acid
Stomach acid reflux
S26 OR ... S87
CONCEPT FOUR: CANCER BIOLOGY OUTCOMES
Proliferation
Reactive Oxygen Species
ROS
Reactive Oxygen Species Production
ROS production
DNA damage
Colony Formation
Apoptosis
Ca^{2+} dependent cell death
Calcium dependent cell death
S89 OR S90 OR S91 OR S92 OR S93 OR S94 OR S95 OR S96 OR S97 OR S98
CONCEPT FIVE: STUDY DESIGN
Randomised controlled trial
Randomized control trial
Cohort study
Prospective cohort study

Retrospective cohort study
Case control study
Cell study
Cellular study
Experimental study
Laboratory study
S100 OR S101 OR S102 OR S103 OR S104 OR S105 OR S106 OR S107 OR S108 OR S109
ALL FIVE CONCEPTS MERGED
S17 AND S25 AND S88 AND S99 AND S110

Table S2.A. Summary of the Concentrations and Durations of Exposure of Bile Acids (Unconjugated Primary and Unconjugated Secondary Bile Acids) Used in the Studies of the Present Systematic Review, compared with Bodily Levels.

Study Authors	Year	Bile Acid Used	Concentration Used (μM)	Duration of Exposure	Mean Serum Concentration (μM)	Mean Concentration in Gastric Juice from Bile Reflux (μM)	Median Barrett's Oesophagus Refluxate Concentration (μM)	Observation
UNCONJUGATED PRIMARY BILE ACIDS								
Matsuzaki et al.	2013	CA	**200**	24 hours	0.287	2.56	25	All concentrations used were higher than bodily levels.
Matsuzaki et al.	2013	CDCA	**10**	24 hours	0.563	0.12	N/A	All concentrations used were higher than bodily levels.
Shan et al.	2013	CDCA	**400**	Not stated	0.563	0.12	N/A	All concentrations used were higher than bodily levels.
UNCONJUGATED SECONDARY BILE ACIDS								
Yamada et al.	2014	DCA	**10, 50 or 100**	6 hours	0.701	3.58	<0.25	All concentrations used were higher than bodily levels.
Tamagawa et al.	2015	DCA	**50, 100, 200**	Up to 12 hours	0.701	3.58	<0.25	All concentrations used were higher than bodily levels.
Xu et al.	2019	DCA	**100**	12 months	0.701	3.58	<0.25	All concentrations used were higher than bodily levels.
Yamada et al.	2014	DCA	**100**	6 hours	0.701	3.58	<0.25	All concentrations used were higher than bodily levels.
Wang et al.	2018	DCA	**100, 300 and 500**	24, 48, 72 and 96 hours	0.701	3.58	<0.25	All concentrations used were higher than bodily levels.
Carrossini et al.	2021	DCA	**200**	30 minute intervals	0.701	3.58	<0.25	All concentrations used were higher than bodily levels.
Quilty et al.	2021	DCA	**200**	6 hours	0.701	3.58	<0.25	All concentrations used were higher than bodily levels.
Feng et al.	2017	DCA	**200**	30 minutes	0.701	3.58	<0.25	All concentrations used were higher than bodily levels.
Feng et al.	2017	DCA	**200**	12 hours	0.701	3.58	<0.25	All concentrations used were higher than bodily levels.
Chen et al.	2020	DCA	**250**	0, 3, 6 and 12 hours	0.701	3.58	<0.25	All concentrations used were higher than bodily levels.
Huo et al.	2020	DCA	**250**	5 minutes	0.701	3.58	<0.25	All concentrations used were higher than bodily levels.
Shan et al.	2013	DCA	**400**	Not stated	0.701	3.58	<0.25	All concentrations used were higher than bodily levels.
Taddei et al.	2014	DCA	**1**	24 hours	0.701	3.58	<0.25	The concentration used was higher in 2/3 bodily measurements.
Huo et al.	2020	UDCA	**250**	5 minutes	0.147	0.03	N/A	All concentrations used were higher than bo
Peng et al.	2014	UDCA	**250**	0 and 8 weeks	0.147	0.03	N/A	All concentrations used were higher than bodily levels.
Banerjee et al.	2016	UDCA	**13 to 15 mg / kg / day**	Lasted for six months	0.147	0.03	N/A	No comparison can be made.

Table S2.B. Summary of the Concentrations and Durations of Exposure of Bile Acids (Conjugated Primary and Secondary Bile Acids and Bile Acid Mixtures) Used in the Studies of the Present Systematic Review, compared with Bodily Levels.								
Study Authors	**Year**	**Bile Acid Used**	**Concentration Used (μM)**	**Duration of Exposure**	**Mean Serum Concentration (μM)**	**Mean Concentration in Gastric Juice from Bile Reflux (μM)**	**Median Barrett's Oesophagus Refluxate Concentration (μM)**	**Observation**
CONJUGATED BILE ACIDS								
Green et al.	2014	TDCA	**0, 50, 100, 400 and 1,000**	2 x 10-minute treatment per day for 3 days	0.061	38.09	5.5	All concentrations used were higher than bodily levels.
Shan et al.	2013	TCA	**1,000**	24 hours	0.071	126.14	39	All concentrations used were higher than bodily levels.
Shan et al.	2013	GCA	**1,000**	24 hours	0.301	266.03	27	All concentrations used were higher than bodily levels.
Liu et al.	2018	TCA	**100**	Not stated	0.071	126.14	39	The concentration used was higher in 2/3 bodily measurements.
Liu et al.	2018	GCA	**100**	Not stated	0.301	266.03	27	The concentration used was higher in 2/3 bodily measurements.
Kanai et al.	2019	TCA	**0, 50, 100, 500 and 1,000**	48 hours	0.071	126.14	39	Some concentrations were higher than bodily levels
Green et al.	2014	TCA	**0, 50, 100, 400 and 1,000**	2 x 10 minute treatment per day for 3 days	0.071	126.14	39	Some concentrations were higher than bodily levels
Green et al.	2014	TCDCA	**0, 50, 100, 400 and 1,000**	2 x 10 minute treatment per day	0.137	157.49	N/A	Some concentrations were higher than bodily levels
Green et al.	2014	GCA	**0, 50, 100, 400 and 1,000**	2 x 10 minute treatment per day for 3 days	0.301	266.03	27	Some concentrations were higher than bodily levels
Green et al.	2014	GCDCA	**0, 50, 100, 400 and 1,000**	2 x 10 minute treatment per day for 3 days	0.931	279.35	N/A	Some concentrations were higher than bodily levels
BILE ACID MIXTURES								
Jiang et al.	2016	Mixture of TCA and GCA in equal	**Not stated**	0, 6, 8, 12 and 24 hours	TCA = 0.071	TCA = 126.14	TCA = 39	No comparison can be made.
					GCA = 0.301	GCA = 266.03	GCA = 27	No comparison can be made.
Bus et al.	2014	DCA + GCA + TCDCA	**Not stated**	24 hours	DCA = 0.701	DCA = 3.58	DCA = <0.25	No comparison can be made.
					GCA = 0.301	GCA = 266.03	GCA = 27	No comparison can be made.
					TCDCA = 0.137	TCDCA = 157.49	N/A	No comparison can be made.

Supplemental Tables S2.A. and S2.B.: Summary of the Concentrations and Durations of Exposure of Neutral-pH, Bile Acids used in the Present Systematic Review, compared with Bodily Levels

CA = cholic acid; CDCA = chenodeoxycholic acid; DCA = deoxycholic acid; UDCA = ursodeoxycholic acid; TDCA = taurodeoxycholic acid; TCA = taurocholic acid; GCA = glycocholic acid; TCDCA = taurochenodeoxycholic acid; GCDCA = glycochenodeoxycholic acid

Supplementary figure S1. PRISMA 2020 checklist for systematic reviews

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	Title
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	Abstract
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	Introduction
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	Introduction
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	Methods
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	Methods
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	Supplementary Material
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	Methods
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	Methods
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	Methods
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	Methods
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	Methods
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	Methods
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	Methods
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	Methods
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	Methods
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	Methods
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	Methods
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	Methods
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	Methods
Certainty	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	Methods

Section and Topic	Item #	Checklist item	Location where item is reported
assessment			
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	Results
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	Results
Study characteristics	17	Cite each included study and present its characteristics.	Results
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	Results
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	Results
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	Results
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	Results
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	Results
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	Results
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	Results
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	Results
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	Discussion
	23b	Discuss any limitations of the evidence included in the review.	Discussion
	23c	Discuss any limitations of the review processes used.	Discussion
	23d	Discuss implications of the results for practice, policy, and future research.	Discussion
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	PROSPERO protocol
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	PROSPERO
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	PROSPERO
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	Main manuscript
Competing interests	26	Declare any competing interests of review authors.	Main manuscript
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	Main manuscript

PRISMA 2020 Checklist

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